Cell, Vol. 22, 545–553, November 1980 (Part 2), Copyright © 1980 by MIT

The White Pock (μ) Mutants of Rabbit Poxvirus. III. Terminal DNA Sequence Duplication and Transposition in Rabbit Poxvirus

Richard W. Moyer,* Ramona L. Graves and Charles T. Rothe
Department of Microbiology
Vanderbilt University School of Medicine
Nashville, Tennessee 37232

Summary

The restriction fragment patterns of the DNA of three white pock (μ) nonhost range mutants (RPμ mutants) of rabbit poxvirus (RPV) show the presence of net insertions of DNA. Two of the mutants (RPμ21 and RPμ7) contain two molar quantities of 13×10^6 and 15×10^6 daltons, respectively, of the left-most viral DNA sequences. The extra copy of these sequences was inserted into the right-most region of the viral genome which in both cases had been modified by deletion of 10×10^6 daltons of the original extreme right-most DNA sequences. Hence two mutants with "left-hand" mirror image termini of 13×10^6 and 15×10^6 daltons of DNA were generated with an overall net increase in mass of 3×10^6 and 5×10^6 daltons of DNA to yield genome molecular weights of 121×10^6 and 123×10^6 for RPμ21 and 7, respectively. A third mutant (RPμ81g) contained 28×10^6 daltons of the right-most parental DNA sequences inserted into the left end of the genome, which had deleted 6×10^6 daltons of the extreme left-hand sequences. This variant contains "right-hand" mirror image termini of 28×10^6 daltons of DNA and a net increase in molecular weight of 22×10^6 daltons of DNA to yield a genome molecular weight of 140×10^6. The cross-linked end of the transposed sequences is retained in all cases. Our data suggest that the terminal DNA sequences at either end of the virus are not essential for a productive infection in certain host cells and that the pathogenicity of orthopoxviruses may be related in part to a series of varied and interchangeable DNA sequences located at both extremes of a highly conserved genome core.

Introduction

The growth of rabbit poxvirus (RPV) on the chorioallantoic membrane (CAM) of embryonated chicken eggs normally results in the production of red ulcerated lesions (pocks) with haemorrhagic centers. "White pock" (μ) variants of RPV arise with a 1% frequency and are recognized as grey-white lesions on the CAM. Most white pock mutants selected in this fashion exhibit a normal wide host range in cultured cells (RPμ mutants), although some (30%) have a drastically reduced host range (RPμhr mutants) (Gemmell and Fenner, 1960). A preliminary analysis of the restriction fragments of the DNA of several individual RPμ mutants revealed that some restriction fragments exhibited a slower electrophoretic mobility than the corresponding fragment of RPV DNA, which suggested that these mutants contain net insertions of DNA. The detailed examination of some of these mutants is hampered by their genetic instability; however, two mutants were sufficiently stable to permit detailed investigation and the construction of conclusive genetic maps. A third mutant was sufficiently stable for only a partial analysis; however, the data are adequate to propose a highly probable gene arrangement. Our results show that extensive regions of either the left or right terminal DNA sequences may be transposed to the opposite end of the genome to replace some preexisting sequences which are deleted. The end result is to generate genetic variants that contain either two left-hand or two right-hand mirror image ends that can comprise up to 33% of the overall length of the genome.

* To whom requests for reprints should be addressed.

Results

The Hind III Sites in the DNA of RPV and the RPμ Mutants

The electrophoretic patterns of the larger restriction fragments generated by Hind III digestion of the DNA of RPV and the three RPμ mutants together with an analysis of these digests for fragments bearing cross-linked ends (Geshelin and Berns, 1974), a procedure used to identify the two terminal fragments (Wittek et al, 1977; McCarron et al., 1978; Archard and Mackett, 1979; Mackett and Archard, 1979; Moyer and Rothe, 1980) are shown in Figure 1. As expected from the known restriction maps of RPV (Figure 2), an analysis of the Hind III fragments of RPV shows that the left-most Hind III B fragment and the right-most Hind III C fragments are cross-linked (Figure 1). The native digestion patterns for both the mutants RPμ21 and 7 show that the original right-most Hind III C fragment is missing and is replaced by a fragment of higher molecular weight. Although only the large Hind III fragments of DNA are shown in the figure, no other fragments in the pattern are altered (data not shown). An analysis of the digest of the mutants for the presence of the terminal fragments shows that the normal left-most Hind III B fragment is cross-linked and that the new higher molecular weight fragment in each case contains the right-hand terminal cross link. From the data the new fragment in both RPμ21 and 7 would appear to be derived from the original Hind III C fragment by a net insertion of 3×10^6 and 5×10^6 daltons of DNA, respectively.

The third mutant, RPμ81g, in contrast lacks the original left-hand terminal Hind III B fragment of the parental virus, and the missing fragment appears to be replaced by a single new fragment of higher mo-

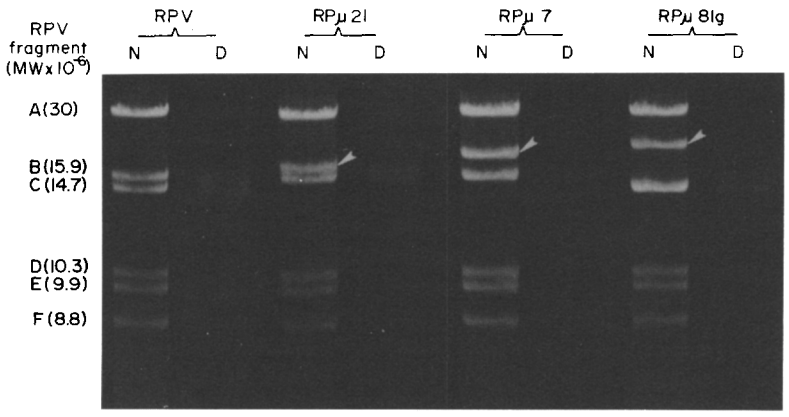

Figure 1. The High Molecular Weight Hind III DNA Restriction Fragments of RPV and the RPμ Mutants after Separation on 0.6% Agarose Gels

(N) Native digests. (D) Digests denatured in 60% (v/v) formamide and rapidly cooled prior to electrophoresis; a procedure in which only cross-linked terminal fragments remain on the gel (Moyer and Rothe, 1980). (↓) The single fragment in each mutant showing a slower electrophoretic mobility.

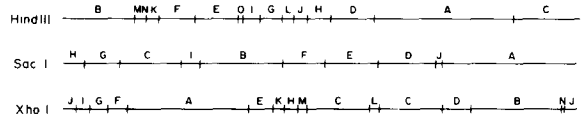

Figure 2. The Hind III, Sac I and Xho I Restriction Maps of RPV DNA (Wittek et al., 1977; Mackett and Archard, 1979)

lecular weight (Figure 1). However, when the Hind III fragments of RPμ81g are examined for the presence of the terminal cross links, one observes that only the right-hand cross-linked Hind III C fragment remains (Figure 1). A densitometric evaluation of the large (Figure 1) and small (data not shown) DNA fragment patterns of RPμ81g also reveals that unlike RPV or the other two RPμ mutants just described, the Hind III C fragment is present in RPμ81g in two molar quantities (Figure 3; Table 1). The DNA of RPμ81g, therefore, contains an additional copy of at least the rightmost Hind III C fragment which appears to be located now at the far left-hand end of the molecule as well to generate "right-hand" mirror image cross-linked ends. In this process the Hind III B fragment has also been altered by a net insertion of 7×10^6 daltons of DNA to create a fragment of higher molecular weight.

The Sac I Sites in the DNA of RPV and the RPμ Mutants

The densitometric scans of the separated DNA fragments generated by Sac I digestion of the DNA of RPV and the RPμ mutants are shown in Figure 4 and Table 1. The RPμ21 and 7 patterns show that both mutants, unlike RPV, contain the left-most adjacent Sac I H and G fragments of RPV in two molar quantities. An analysis of the fragments for the presence of the terminal cross links shows, as expected from the maps (Figure 2), that the Sac I A and H fragments of RPV are cross-linked, but that only the Sac I H fragment of the mutants RPμ21 and 7 appears to contain a cross link (data not shown). Since the Hind III digests (Figure 1) show that each mutant contains two cross-linked termini, this result suggests that the left-most Sac I H fragment of RPV has been duplicated and is now present at each end of both mutant genomes. Fur-

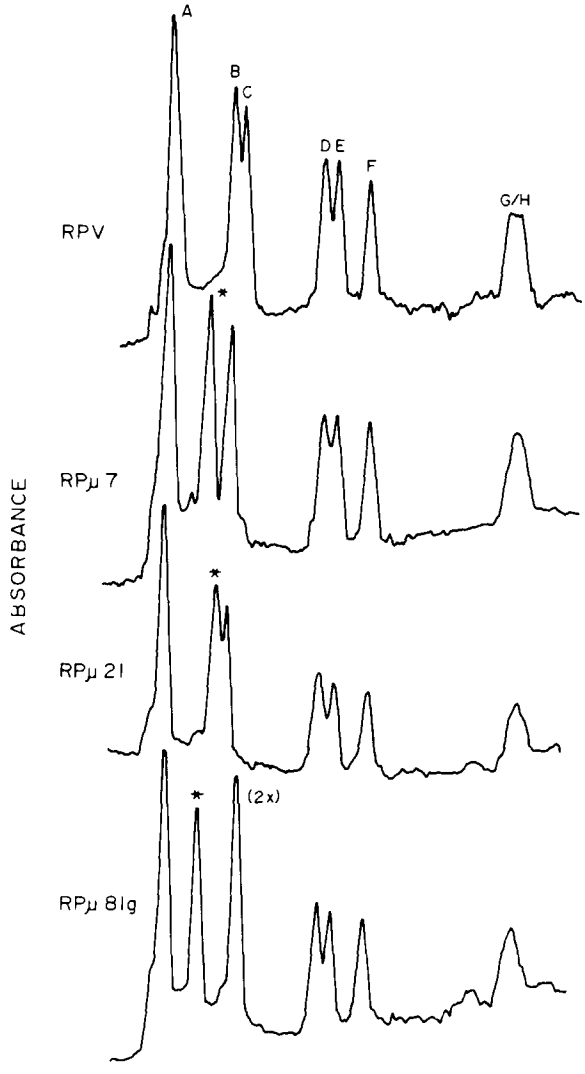

Figure 3. Densitometric Evaluation of the Native Hind III Restriction Digests Showing the Two Molar (2x) Presence of Hind III C Fragment in RPμ81g

The Hind III digests of the mutants shown in Figure 1 were scanned with a Jocye Lobel densitometer. The altered Hind III C fragments of RPμ21 and 7 and the altered Hind III B fragment of RPμ81g are indicated by asterisks.

Table 1. The Molar Ratios of Restriction Fragments in RPV and the RPμ Mutants

Hind III[a]

	Fragment							
Virus	A	B	C	D	E	F[e]	G/H	New[c]
RPV	1.0	1.1	0.9	1.1	1.0	1.0	1.0	None
RPμ7	1.0	1.0	—[d]	1.2	0.9	1.0	0.9	1.0
RPμ21	1.0	1.0	—	1.2	1.0	1.0	0.9	1.2
RPμ81g	1.0	—	1.8	1.1	1.0	1.0	0.9	0.9

Sac I[b]

	Fragment								
Virus	A	B	C/D	E	F[e]	G	H	I	New[c]
RPV	1.2	1.3	1.0	1.0	1.0	1.2	1.3	1.0	None
RPμ7	—	1.2	1.0	1.3	1.0	1.8	1.9	1.1	1.2
RPμ21	—	1.1	0.9	1.0	1.0	1.8	2.0	0.9	1.2
RPμ81g	1.7	0.8	0.9	1.0	1.0	—	—	0.9	None

[a] Quantitation of scans shown in Figure 3.
[b] Quantitation of scans shown in Figure 4.
[c] Fragment bearing asterisk in either Figure 3 or Figure 4.
[d] (—) Missing parental fragment.
[e] Unimolar fragment upon which calculations are based.

thermore, the Sac I restriction patterns of these mutants show that the Sac I A fragment from the far right-hand end of the molecule is somewhat shortened compared to the corresponding Sac I A fragment of RPV. We propose that the Hind III and Sac I DNA digestion patterns of the mutants RPμ21 and 7 are best explained by the transposition and insertion of 13×10^6 and 15×10^6 daltons of DNA, respectively, including sequences contained in the Sac I G and H fragments, from the left-hand region of the RPV genome into the far right-hand end, which has been additionally modified in both mutants by the deletion of about 10×10^6 daltons of DNA. Compared to RPV, the net increase in genome molecular weight would be 3×10^6 for RPμ21 and 5×10^6 for RPμ7. This results in the generation of large left-hand mirror image termini in the genomes of these mutants.

The Sac I digestion pattern of RPμ81g, in contrast, shows that the material in the Sac I A region is present in two molar amounts (Figure 4 and Table 1) and that only material in this region is cross-linked (data not shown). In addition, the left-most adjacent Sac I H and G fragments, present in RPV DNA, are missing in RPμ81g. The Hind III and Sac I DNA patterns of RPμ81g can be explained by the transposition and insertion of 28×10^6 daltons of DNA from the far right-hand end into the far left-hand end of the genome which has been also modified by a deletion of 6×10^6 daltons of DNA. There would be a net increase of 22×10^6 daltons in the molecular weight of the DNA of this mutant compared to that of RPV. The cleavage of such a variant with Hind III would generate a two molar quantity of the Hind III C fragment and a Hind III B fragment of significantly increased molecular weight as we have observed (Figure 3). The end result of this transposition is the generation of a mutant DNA that contains 28×10^6 dalton right-hand mirror image termini.

On the basis of the data described above Xho I restriction maps of the three mutants and RPV have been proposed and are presented in Figure 5. We have also indicated in this figure the Hind III sites that would account for an altered Hind III C fragment of increased mass for mutants RPμ21 and 7. Similarly we have included the pertinent Hind III sites in RPμ81g which would allow for the generation of two molar quantities of the normal Hind III C fragment of RPV DNA as well as an altered Hind III B band of increased mass.

Double Digestion of the Altered Hind III DNA Fragments of the RPμ Mutants

It is the purpose of this and the following section to present data supporting our hypothesis that DNA sequences originally present on one end of the genome can be transposed to the other end of the genome, by confirming the maps presented in Figure 5, and if possible by further defining the limits of the transpositions. The altered Hind III C fragments of RPμ21 and 7 (Figure 1), which carry the right-most terminal cross link and an apparent insertion of DNA, were isolated and redigested with Xho I, and the results are shown in Figure 6. Fragments are generated by Xho I digestion of the Hind III C fragment of RPμ21 which comigrate with the Xho I G, I and J fragments (Figure 6B). The Xho I G and I fragments must have originated

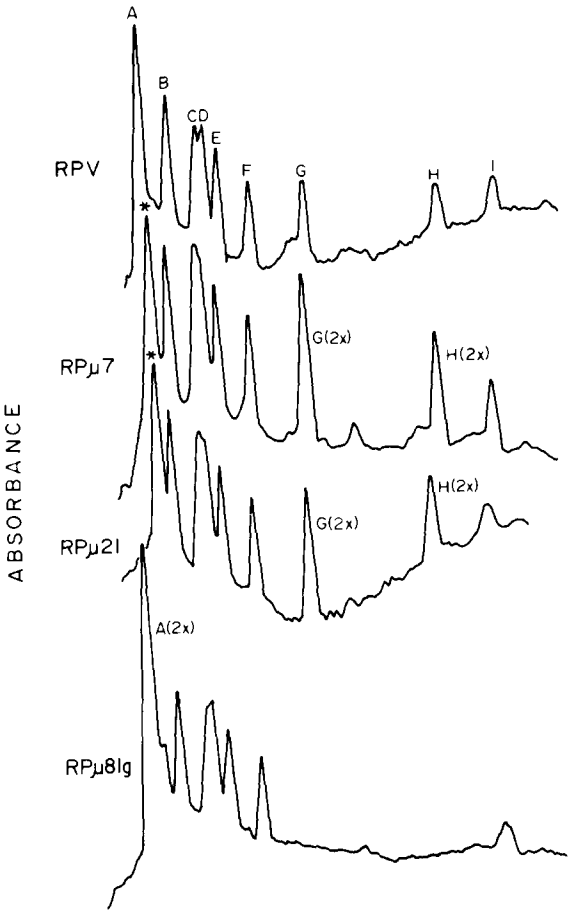

Figure 4. Densitometric Evaluation of the Native Sac I Digests of the DNA of RPV and the RPμ Mutants after Separation on 0.6% Agarose Gels

The restriction fragments resulting from the Sac I digest of the DNAs were quantitated by densitometric scanning as described in the legend to Figure 3. The bands present in two molar quantities (2×) are shown in the figure. The shortened Sac I A fragments of RPμ21 and 7 are indicated by an asterisk.

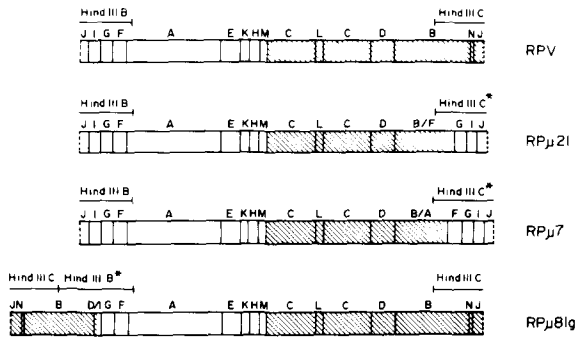

Figure 5. The Proposed Xho I Maps of the DNA of RPV and of the RPμ Mutants 21, 7 and 81g

The right and left halves of the parental RPV genome are arbitrarily distinguished by crosshatching to more readily demonstrate the original source of the sequences in each of the mutant DNAs. In addition, for reference the position of pertinent Hind III fragments is included as discussed in the text. Hind III fragments of increased molecular weight in the mutants which result after transposition are indicated by an asterisk.

from the left-hand end of the genome. A fourth fragment of higher molecular weight is also seen which we propose results from the fusion of parts of the left-hand Xho I F and right-hand Xho I B fragments. Similarly, when the altered Hind III C fragment of RPμ7 is redigested with Xho I, fragments are generated which co-migrate with the Xho I fragments F, G, I and J (Figure 6A). The Xho I fragments F, G and I could only have originated from the left-hand region of the genome. The presence of an intact Xho I F fragment in RPμ7 is consistent with the more extensive transposition of left-hand sequences predicted in Figure 5 for RPμ7. We would have further predicted for RPμ7 a fusion fragment consisting of portions of the left-hand Xho I A and the right-hand Xho I B fragment which is not apparent in the stained gel. However, autoradiography of the separated ^{32}P-labeled digest reveals an extensive family of bands near the top of the gel in the molecular weight range expected for the fusion fragment(s). This family of fragments serve to emphasize the intrinsic instability of this particular mutant. Later passages of both cloned and uncloned stocks of RPμ7 showed deletions of most of the transposed material and further work with this mutant was not possible.

In the interpretation of the Xho I digestion pattern of the altered Hind III B fragment of mutant RPμ81g, it must be remembered that a large part of the sequences initially transposed from the right to the left end have been eliminated from the left hand during the initial Hind III digestion leading to the formation of a two molar Hind III C fragment (Figures 3 and 5). The transposed sequences that remain inserted in the altered Hind III B fragment should, therefore, be derived from the right-hand Xho I D and B fragments plus the original unaffected left-hand Xho I G and F fragments and a small region of the Xho I A fragment (Figure 5). Redigestion of the altered Hind III B fragment with Xho I (Figure 6C) yields, in fact, the intact Xho I fragments F and G, which delineate that portion of the original Hind III B fragment unaffected by deletion, a small Xho I D/I hybrid fragment near the bottom of the gel plus that portion of the Xho I B fragment (migrating near the top of the gel) that remains after the removal of the Hind III C fragment in the initial digest. The size of the predicted remaining portion of the Xho I A fragment is very small and it would migrate off the gel under the conditions used here. In other experiments, direct Xho I restriction analyses of the mutant DNA genomes show that the expected end fragments (RPμ21-G, I, J; RPμ7-F, G, I, J; and RPμ81g-B, N, J) are present in two molar amounts (data not shown). We conclude from these data that both ends of the RPV DNA contain sequences that can be transposed.

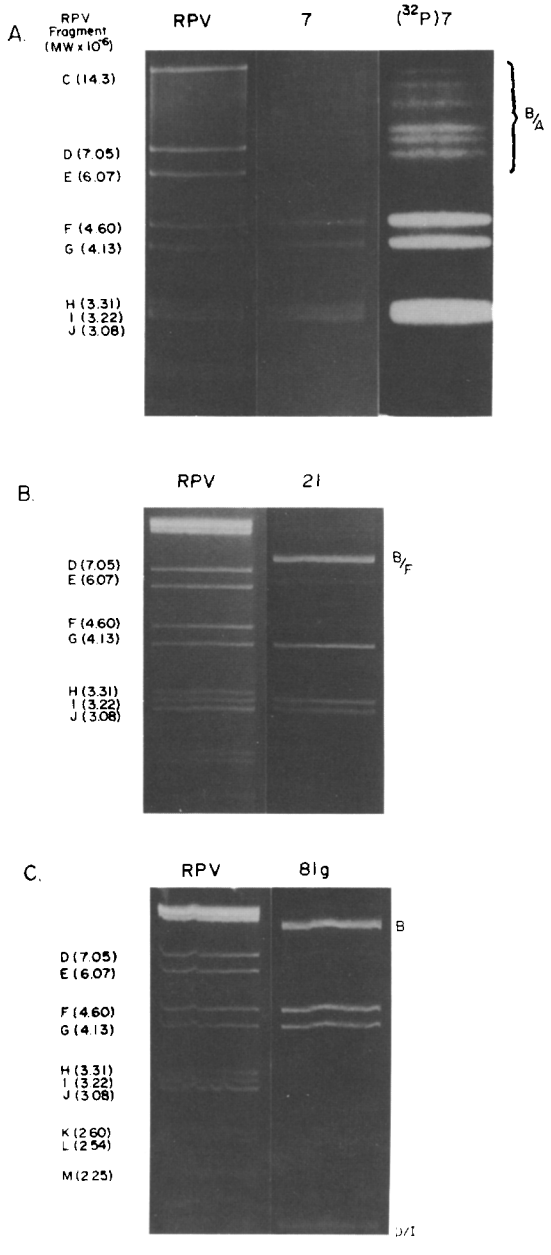

Figure 6. Redigestion of the Altered Hind III B or C Fragments of the RPμ Mutants

Digests of the DNA of the RPμ mutants were prepared and the fragments were separated by electrophoresis on 0.6% agarose gels that contained a single continuous sample well. The altered Hind III B fragment of RPμ81g and the altered Hind III C fragments of RPμ21 and 7 were eluted electrophoretically from the gel, concentrated by ethanol precipitation and redigested with Xho I. The fragments generated by the Xho I redigestion were separated by electrophoresis on 1.2% agarose gels and stained with ethidium bromide. The electrophoretic mobilities were compared in each case to an Xho I digest of total RPV DNA. The Xho I digestion of the altered Hind III C fragment of RPμ7 and of RPμ21 is shown in (A) and (B) respectively; whereas the Xho I digestion of the altered Hind III B fragment of RPμ81g is shown in (C). The probable origin of fragments generated by Xho I redigestion of the Hind III fragment not corresponding to parental Xho I fragments is indicated and discussed in the text. The third panel for RPμ7 in (A) is an autoradiogram of the stained gel shown in the middle panel.

The Hybridization of the Altered ^{32}P-Labeled Hind III Fragments of the RPμ Mutants to Separated Immobilized Xho I Fragments of RPV DNA

A prediction stemming from the results presented above is that sequences present at the right-hand termini of RPμ21 and 7 should hybridize with the Xho I fragments F, G and I which are unique to the left-hand terminus of the parental RPV genome. A test of this proposal was not possible for RPμ7 because the instability of this mutant, noted in Figure 6, led to the rapid elimination of sequences essential for this experiment. We were, however, able to test this hypothesis with the other two mutants. The ^{32}P-labeled DNA of RPμ21 was purified and digested with Hind III, and the fragments were separated by electrophoresis on agarose gels. The altered Hind III C fragment of RPμ21 was isolated from the gel and was then hybridized to the separated Xho I fragments of RPV DNA which were immobilized on nitrocellulose strips. As a control we compared the hybridization of the isolated ^{32}P-labeled Hind III C fragment derived from RPV. The ^{32}P-labeled right-most Hind III C fragment of RPV hybridizes, as expected, only to the right-hand Xho I fragments B and J (Figure 7E). The altered right-most Hind III C fragment of RPμ21, however, hybridizes not only to the Xho I B and J fragments but also to the Xho I fragments F, G and I which are located in the left-most region of the genome of RPV (Figure 7D). With the altered fragment of RPμ21 we also observe a small amount of anomalous hybridization to the Xho I A and C fragments; however, the RPV Hind III B control fragment of RPV (Figure 7C), which in fact contains most of the transposed sequences of RPμ21, also shows some hybridization to Xho I fragments A, B and C. It is possible that sequences only within the wild-type Hind III B fragment are repeated at these other locations in the genome as this anomalous hybridization is not seen with the Hind III C control fragment of RPV (Figure 7E). It is clear, therefore, that RPμ21, and by analogy based on the data of Figure 6, RPμ7, have duplicated 13×10^6 and 15×10^6 daltons of DNA, respectively, from the left-hand region of the genome and inserted this DNA into the far right of the molecule which has deleted about 10×10^6 daltons of the original right-most terminal sequences.

A similar but reciprocal situation exists in the case of RPμ81g which involves the opposite end of the molecule. Again it must be pointed out that according to our model for the gene arrangement of RPμ81g (Figure 5), the altered Hind III B fragment is not the actual left-most fragment in this mutant. Both ends consist of Hind III C fragments. Therefore, the altered Hind III B fragment which was used here for hybridization should contain remnants both of the original RPV Hind B fragment (that is, the Xho I fragments G, F and a small portion of I and A) and the more internal regions of the right-hand transposed end (that is, portions of the Xho I B and D fragments). We tested

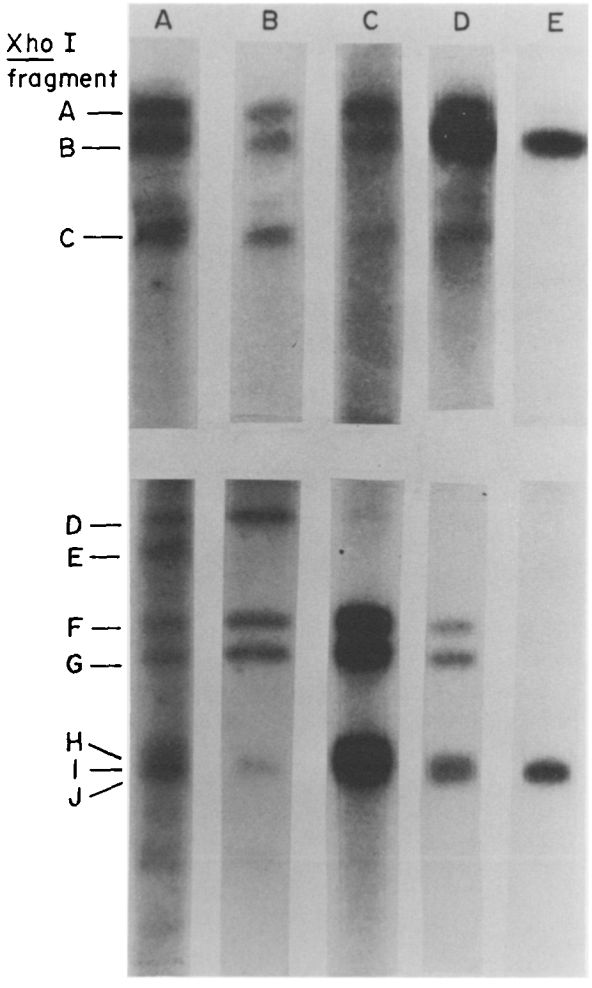

Figure 7. The Hybridization of ^{32}P-Labeled Altered Hind III Fragments of RPV and the RPμ Mutants to Separated Xho I Fragments of RPV DNA Immobilized on Nitrocellulose Strips

The DNAs of RPV and of the RPμ mutants were digested with Hind III and the fragments were separated by electrophoresis on a 0.6% agarose gel that contained a single continuous sample well. After electrophoresis and staining of the gel with ethidium bromide, the bands were excised and the DNA was eluted electrophoretically from the agarose. The hybridization of labeled DNA (6,000–30,000 cpm) to nitrocellulose strips containing the Xho I fragments of RPV DNA was as described by Ficht and Moyer (1980). The representative position of the Xho I fragment digest is shown by the hybridization of unfractionated total ^{32}P-labeled RPV DNA in panel (A). The hybridization pattern for the altered Hind III B fragment of RPμ81g is shown in panel (B), for the Hind III B fragment of RPV in panel (C), for the altered Hind III C fragment of RPμ21 in panel (D) and for the Hind III C fragment of RPV in panel (E).

this hypothesis by digesting ^{32}P-labeled RPμ81g DNA with Hind III, isolating the separated altered Hind III B fragment from agarose gels, followed by hybridizing this fragment to separated Xho I fragments of RPV DNA immobilized on nitrocellulose strips. As a control the ^{32}P-labeled Hind III B fragment of RPV DNA hybridizes predominantly, as expected, to the Xho I fragments J, I, G, F and A (Figure 7C). The altered Hind III B fragment of RPμ81g hybridizes as predicted to the Xho I B, D, I, G, F and A fragments (Figure 7B). Significant hybridization to the Xho I B and D fragments of RPV DNA offers further support to the proposed gene arrangement for RPμ81g shown in Figure 5. Again, we found hybridization to the Xho I C fragment of RPV DNA. DNA in the Xho I C region is derived from two co-migrating fragments of DNA to the left of the Xho I B region of RPV DNA (Figure 5). One possible explanation for this result, as discussed above, is that left-end sequences of both the mutant and wild-type DNAs are repeated in one or both of the co-migrating Xho I C fragments.

In data not shown here, the unaltered Hind III C band of RPμ81g and the Hind III B band of RPμ21 gave the same hybridization patterns as the control Hind III C and B fragments of RPV DNA, respectively (Figures 7C and 7E). In summary, all our data are consistent with the genetic maps proposed in Figure 5 and indicate that transpositions of extensive regions of DNA from either end to the other are possible.

The Origin of the RPμhr Mutants of RPV

If one randomly selects white pock variants of RPV, 30% have a drastically reduced host range (the RPμhr mutants) as compared to the remaining 70% which are RPμ mutants and have normal host range properties. In earlier studies we presented maps for several mutants of the RPμhr class, one of which was designated RPμhr8sm. The data on this mutant initially suggested that it arose as a simple deletion of DNA from the left-hand region of the genome (Moyer and Rothe, 1980). One reason for originally mapping RPμhr8sm was the fact that it was isolated by us as a stable segregant during the purification of the nonhost range mutant RPμ81g (mapped here). The host range mutant elaborates a white pock on the CAM smaller than that of the RPμ81g parent. The Xho I restriction maps of RPV, RPμ81g and RPμhr8sm are presented in Figure 8. It can be seen that RPμhr8sm must have originated from wild-type virus not by simple deletion but instead from RPμ81g by the subsequent deletion of both the transposed DNA and some additional sequences from the original left-hand end. Virtually all evidence of the original transposition of sequences from the right-hand end to the left end of the parental RPμ81g has been eliminated during the subsequent segregation which forms the stable RPμhr8sm derivative.

Discussion

We have presented data for three unique rabbit poxvirus variants, each of which contains a major rearrangement of sequences in the terminal regions of the genome. Two mutants (RPμ21 and 7) have deleted 10 $\times$ 10^6 daltons of DNA from the right-most region of the genome. The deleted sequences are replaced by 13 $\times$ 10^6 (RPμ21) or 15 $\times$ 10^6 (RPμ7) daltons of DNA

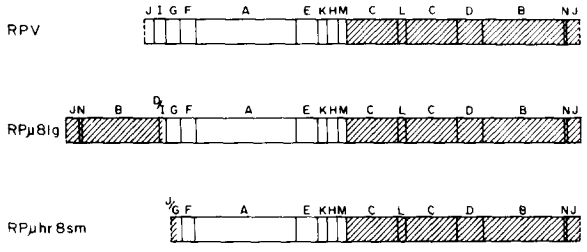

Figure 8. The Xho I Maps of RPV, RPμ81g and RPμhr8sm

The mutant RPμhr8sm (Moyer and Rothe, 1980) was isolated as a segregant of RPμ81g by virtue of the fact that it elaborates a much smaller white pock on the CAM than its parent RPμ81g. The left and right halves of the parental RPV molecule have been arbitrarily distinguished for clarity by crosshatching.

from the left-most region of the genome to generate variants with left-hand mirror image ends. The third mutant RPμ81g has replaced 6 × 10^6 daltons of deleted left-most sequences by 28 × 10^6 daltons of right-hand sequences to generate a variant with right-hand mirror image ends.

We have also shown that at least one of the white pock host range mutants (RPμhr8sm, for example), which superficially appears to result from a simple deletion of left-hand sequences, arises instead from a white pock nonhost range transposition mutant (RPμ81g) by the subsequent loss of both the transposed right-hand sequences and some additional remaining left-hand sequences (Figure 8). If one compares the restriction maps of RPμ81g and RPμhr8sm, it appears that the emergence of the host range phenotype in cell culture can be localized to the right portion of the Xho I I and left-most region of Xho I G fragments, since deletion within this region leads to the loss of the ability to grow on pig kidney cells. The overall frequency of generation of white pock variants in a wild-type population is high (1%), and since the appearance of the white pock phenotype to date is the only measure of terminal transposition frequency that we have, the interchange of termini may be quite a common event in poxviruses. Furthermore, the deletion of sequences often appears to continue in the transposition mutants until a stable variant emerges which very often has deleted functions needed for productive infections in certain hosts and results in the formation of the host range mutants.

The genome molecular weight of the largest transposition mutant (RPμ81g) is 140 × 10^6, while that of parental RPV is 118 × 10^6. Earlier we reported that of the several white pock host range mutants, one, RPμhr23, showed a net loss of 20 × 10^6 daltons of DNA to yield a genome molecular weight of 98 × 10^6 (Moyer and Rothe, 1980). The generation of viable RPV mutants ranging in molecular weights from 140 × 10^6 to 98 × 10^6 demands that the processes of DNA packaging and assembly of poxvirions must be capable of coping successfully with a viral genome of enormous variability. Our earlier study of the RPμhr

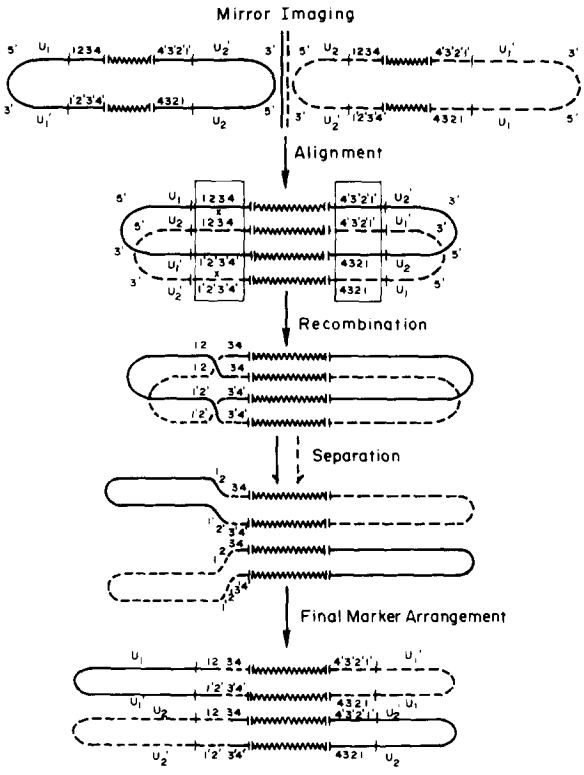

Figure 9. A Model for the Generation of Poxvirus Variants with Duplicated Termini

Two identical parental RPV DNA molecules are presented as mirror images with respect to both axes. The strand polarity is indicated and the molecules depicted have exaggerated ends and abbreviated core regions for illustrative purposes only. The small inverted terminal repeats contained within the two distinguishable terminal regions, designated U_1 and U_2, at the extremes of the molecule are not shown. Further details of the model are given in the text.

mutants showed that net DNA deletions of up to 20 × 10^6 daltons were all located in the left-most region of the genome. The deletions in the left-most region of the DNA lead to profound effects on the host range properties of the virus (Moyer and Rothe, 1980). The data here show that up to 10 × 10^6 daltons of DNA can also be eliminated from the right-most region of the genome, as for example in RPμ21 and 7, without any effect on growth in cell culture. If all these deletions are additive, then 30 × 10^6 daltons or 25% of the viral genome is either nonessential or essential for growth only on certain cells.

We present a molecular model to account for the generation of these variants (Figure 9) which obviates the need for either end to end circularity or concatenated intermediates, forms of poxvirus DNA for which no evidence exists (Esteban and Holowczak, 1977; Esteban et al., 1978). Recent studies by McFadden and Dales (1979) have described poxvirus DNAs with mirror image deletions within the inverted terminal repeats at both ends of the molecule. Such deletions could have arisen from a circular form of viral DNA. We, however, have described deletions within the

internal half of the left-hand inverted terminal repeat which are not mirrored in the opposite end (Moyer and Rothe, 1980). The existence of such deletions within only one of the two inverted terminal repeats suggests to us that any end to end molecular interactions, whether through circular or concatenated intermediates, if they occur at all, may involve only the most distal regions of the inverted terminal repeats. This would be too limited an amount of sequence involvement to account for the extensive regions of DNA which are transposed in the mutants described here.

The RPV DNA shown in the model is depicted with cross-linked ends, but the model applies equally well to molecules where the cross links have been removed, a phenomenon which occurs transiently during the infectious cycle (Pogo, 1977, 1980). We represent the RPV genome in the diagram with an abbreviated core and exaggerated ends to focus on the events at the termini. The regions U_1 and U_2 represent distinguishable and transposable terminal regions of the genome and are made up in small part of the known inverted terminal repeats at the very ends of the molecule, which in the interest of simplicity are not shown. The DNA sequences on the strand complimentary to the U_1 and U_2 regions are represented as U_1' and U_2'. We propose that recognition sequences, such as the inverted repeats 1 2 3 4 and 1′ 2′ 3′ 4′, are present in both the right and left domains of the molecule internal to the regions to be transposed. If a second identical PRV DNA molecule is rotated 180° about both the X and Y axes, one can produce "mirror image" parent molecules. After proper alignment recombination between either of the two sets of the inverted sequences could occur to generate, in one step, a pair of variant DNA molecules, one of which contains left-hand mirror image ends and another that contains right-hand mirror image ends. Although no net loss of DNA occurs between the two molecules by the recombinational event, note that each individual molecule now simultaneously contains a deletion of sequences originally present at the modified end, where the extent of the deletion is determined by the location of the recognition sequences.

The set or sets of recognition sequences, such as inverted repeats, that allow for the transposition event would also lead us to predict that the recombinational points between the old and the new sequences should be relatively constant. Diversity among left- or right-hand mirror images may then arise during the subsequent steps of sporadic deletion that seem to occur frequently in some of these mutants creating on occasion, the host range (RPμhr) mutants. The proposed maps of two left-hand mirror image mutants, RPμ21 and 7, are consistent with a unique cross-over point during transposition, but they represent only two examples. A study of more of these types of mutants with both left- and right-hand mirror image ends is needed to test this model.

Experimental Procedures

Cells and Virus

Wild-type rabbit poxvirus (RPV, Utrecht strain) was obtained from the American Type Culture Collection. The routine propagation of all virus strains was on chicken embryo fibroblasts (CEF). The CEF were prepared from 11 day old embryos and maintained on Gibco medium F-199 supplemented to contain 10% calf serum, 3 mg/ml tryptose phosphate broth, 2 mM glutamine, 100 units penicillin, 100 μg/ml streptomycin and 0.028 M HEPES buffer (pH 7.3) (Moyer and Rothe, 1980).

Selection of RPμ Mutants

Embryonated eggs were incubated 11 days at 37°C. Plaque-purified RPV was resuspended in unsupplemented F-199 and inoculated onto the chorioallantoic membrane of an egg. Routinely, 0.2 ml of diluted virus was added at a concentration sufficient to give 20–25 pocks per egg. After infection the eggs were incubated for 64 hr at 37°C. The eggs were then chilled for 90 min and any egg showing a massive hemorrhage on the CAM was discarded. Individual, well separated white pock variants were picked with a needle and placed into 1 ml unsupplemented F-199, sonicated briefly and reinoculated again onto the CAM of 11 day old embryonated eggs. After the initial selection of a given mutant from a pock, each variant was passed twice more as above on the CAM to observe segregation rates and finally cloned once on CEF cells before stocks were prepared for the isolation of DNA.

Each individual mutant elaborates a characteristic type of white pock on the CAM. Any mutant which by the third cloning shows a continued appearance of pocks with a morphology distinct from the initial isolate at a rate exceeding 1 or 2% was not further purified. Our experience has shown that mutants yielding obvious segregation at rates greater than this are genetically too unstable to yield reproducible DNA restriction patterns in successive preparations of the virus.

Preparation of ^{32}P-Labeled Viral DNA

The medium on slightly subconfluent monolayers (2×10^8 total cells) was replaced by medium lacking phosphate. After incubation for 24 hr the cells were infected with RPV or one of the RPμ mutants in phosphate-free medium and the virus was allowed to adsorb for 2 hr at 37°C. After adsorption phosphate-free medium containing ^{32}P-orthophosphate (30 μCi/ml) was added and the incubation was continued for a total of 24 hr at 37°C. The medium was then removed and replaced by phosphate-buffered saline (PBS). Cells were scraped into the PBS and harvested by low speed centrifugation. The total yield of virus in this procedure is much lower than normal. Therefore prior to the isolation of virus and extraction of the DNA, the cells that contained ^{32}P-labeled virus were combined with 2×10^8 unlabeled "carrier" cells infected with the homologous virus under standard conditions. A total of 300 μg of purified DNA was recovered from a total of 4×10^8 cells at a final specific activity of 1×10^5 cpm/μg of DNA.

General Methods

The methods for the purification of virus from infected cells and the isolation of DNA from purified virus were described previously (Moyer and Rothe, 1980). The procedures for the digestion of viral DNA with the enzymes Hind III, Sac I and Xho I, the separation of the DNA fragments by electrophoresis on agarose gels, the analysis of restriction digests for fragments bearing terminal cross links, the staining of the gels with ethidium bromide, the method of photography of the stained gels and the relative quantitation of the DNA bands have been described earlier (Moyer and Rothe, 1980). Nitrocellulose strips containing the separated and immobilized Xho I restriction fragments of RPV DNA were prepared according to the method of Southern

(1975). The hybridization of the ^{32}P-labeled restriction fragments to the nitrocellulose strips containing the Xho I fragments of RPV DNA was carried out as described by Ficht and Moyer (1980).

Acknowledgments

We would like to acknowledge the comments of Dr. S. A. Moyer on the manuscript. This work was supported by grants from the NIH and the National Science Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

Received June 19, 1980; revised August 19, 1980

References

Archard, L. C. and Mackett, M. (1979). Restriction endonuclease analysis of red cowpox virus and its white pock variant. J. Gen. Virol. *45*, 51–64.

Esteban, M. and Holowczak, J. A. (1977). Replication of vaccinia DNA in mouse L cells. I. *In vivo* DNA synthesis. Virology *78*, 57–75.

Esteban, M., Soloski, M., Cabrera, C. V. and Holowczak, J. A. (1978). Replication of vaccinia virus DNA and studies on the structure of the viral chromosome. Cold Spring Harbor Symp. Quant. Biol. *43*, 789–799.

Ficht, T. and Moyer, R. W. (1980). Isolation and characterization of a putative bacteriophage T5 transcription-replication enzyme complex from infected *Escherichia coli*. J. Biol. Chem. *255*, 7040–7048.

Gemmell, A. and Fenner, F. (1960). Genetic studies with mammalian poxviruses. III. White pock (μ) mutants of rabbit poxvirus. Virology *11*, 219–235.

Geshelin, P. and Berns, K. I. (1974). Characterization and localization of the naturally occurring cross links in vaccinia virus DNA. J. Mol. Biol. *88*, 785–796.

McCarron, R. J., Cabrera, C. V., Esteban, M., McAllister, W. T. and Holowczak, J. A. (1978). Structure of vaccinia DNA: analysis of the viral genome by restriction endonucleases. Virology *86*, 88–101.

McFadden, G. and Dales, S. (1979). Biogenesis of poxviruses: mirror-image deletions in vaccinia virus DNA. Cell *18*, 101–108.

Mackett, M. and Archard, L. E. (1979). Conservation and variation in orthopoxvirus genome structure. J. Gen. Virol. *45*, 683–702.

Moyer, R. W. and Rothe, C. T. (1980). The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions. Virology *102*, 119–132.

Pogo, B. G. T. (1977). Elimination of naturally occurring cross links in vaccinia virus DNA after viral penetration into cells. Proc. Nat. Acad. Sci. USA *74*, 1739–1742.

Pogo, B. G. T. (1980). Changes in parental vaccinia virus DNA after viral penetration into cells. Virology *101*, 520–524.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*, 503–517.

Wittek, R., Menna, A., Schümperli, D., Stoffel, S., Müller, H. K. and Wyler, R. (1977). *Hind* III and *Sst*I restriction sites mapped on rabbit poxvirus and vaccinia virus DNA. J. Virol. *23*, 669–678.