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The Mechanism of Cytoplasmic Orthopoxvirus DNA Replication

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Summary

Orthopoxvirus DNA replication occurs in the cytoplasm of infected cells within discrete foci designated as virosomes. We show that newly synthesized rabbit poxvirus (RPV) virosomal DNA consists predominantly of concatamers wherein unit length molecules are joined by fusion of two left (LL) or right (RR) ends, resulting in genomes aligned in alternating head-to-head and tail-to-tail mirror image arrays. These concatameric molecules serve as the substrates from which unit length DNA molecules are excised during morphogenesis. We propose a mechanism by which internal deletions within these concatameric arrays prior to genome excision and packaging could create inverted terminal repeats and generate gene duplications.

Introduction

All members of the orthopoxvirus family contain a linear double-stranded DNA molecule with crosslinked ends (Geshelin and Berns, 1974). In addition, all members, with the notable exception of variola (Dumbell and Archard, 1980), contain inverted terminal repeats (Garon et al., 1978; Wittek et al., 1978; Mackett and Archard, 1979; Schumperli et al., 1980). Information about the mode of replication of these viruses is limited. It is generally agreed that DNA synthesis is cytoplasmic (Hruby et al., 1979), and it is thought that both initiation and termination of DNA synthesis occur at the termini of the molecule (Esteban et al., 1977; Pogo et al., 1981). Relatively little, however, is known about the molecular intermediates in the replicative process. The presence of inverted terminal repeats in the DNA of most members of this virus family and the existence of a mutant that contains a deletion mirrored in both ends of the inverted terminal repeat (McFadden and Dales, 1979) have led to the suggestion that either circular or linear head-to-tail concatameric molecules serve as intermediates in the overall process of replication (McFadden and Dales, 1979; Archard, 1979). The recent demonstration of tandem repeats within the inverted terminal repeats led to the suggestion that the single strands of viral DNA circularize internally during DNA synthesis (Wittek and Moss, 1980) by analogy with the Sambrook model for adenovirus DNA replication (cited by Daniell, 1976, and Lechner and Kelly, 1977). Although the use of the adenovirus model negates the need for any concatamer formation, successful poxvirus DNA replication would still require noncovalent head-to-tail interactions between the left (L) and right (R) ends of the single-stranded DNA chains (Wittek and Moss, 1980). Two pieces of evidence, however, argue against any obligatory head-to-tail DNA end interactions during replication. First, there are no inverted terminal repeats in variola virus DNA (Dumbell and Archard, 1980) and, second, deletion mutants of rabbit poxvirus exist where a portion of only the left, but not the right, inverted terminal repeat is deleted (Moyer and Rothe, 1980). The data we present suggest that the DNA of the cytoplasmic virosomes is concatameric wherein genomes are joined by the alternating fusion of two left (LL) then two right (RR) terminal fragments. Molecules therefore are joined head-to-head and tail-to-tail, rather than head-to-tail, creating mirror image arrays of genomes. We suggest mechanisms first whereby these concatamers can serve as the source of viral genomes for packaging, and second whereby internal deletions within the concatamers prior to packaging can generate inverted terminal repeats and result in mutants containing large, mirror image terminal gene duplications or deletions. These mechanisms may apply not only to the cross-linked DNA of orthopoxviruses, but also to eucaryotes such as yeast where cross-linked chromosomes have been demonstrated (Forte and Fangman, 1979).

Results and Discussion

Synthesis and Processing of Virosomal DNA

Newly synthesized poxvirus DNA is thought to accumulate within cytoplasmic foci called virosomes, which can be easily isolated from cytoplasmic extracts of infected cells on the basis of their extremely rapid rate of sedimentation (Dahl and Kates, 1970). It has also been suggested that progeny molecules are subsequently excised from virosomal DNA (Joklik and Becker, 1964; Dahl and Kates, 1970). When cells infected with rabbit poxvirus (RPV) are labeled with ^{3}H-thymidine for 1 hr, beginning at the onset of DNA replication (3 hr after infection), most of the cytoplasmic viral DNA initially accumulates in the rapidly sedimenting virosomes (Figure 1A). The viral DNA labeled later (between 4 and 5 hr after infection) is somewhat more heterodisperse (Figure 1B). Although a significant proportion of the total DNA is still located in the virosomes, a second major peak sedimenting near the top of the gradient, but slower than mature marker virus, is also evident. When viral DNA of infected cells is labeled from 5 to 6 hr after infection, most of the newly labeled DNA appears near the top of the gradient (Figure 1C). We find that the DNA at the top of the gradient sediments under neutral conditions faster than purified viral DNA does and, unlike virosomal DNA, is complexed to several major viral structural proteins (data not shown).

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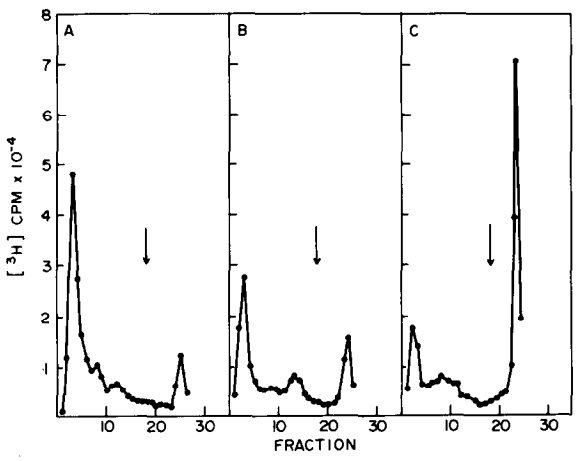

Figure 1. Compartmentalization of Newly Synthesized RPV DNA

The compartmentalization of newly synthesized ^{3}H-RPV DNA was determined by subjecting cytoplasmic extracts to sedimentation in sucrose gradients as described in Experimental Procedures. Samples of infected cells were labeled from 3 to 4 hr (A), 4 to 5 hr (B), and 5 to 6 hr (C) after infection. The sedimentation position of purified virus under these conditions is indicated by an arrow (↓). Sedimentation is from right to left.

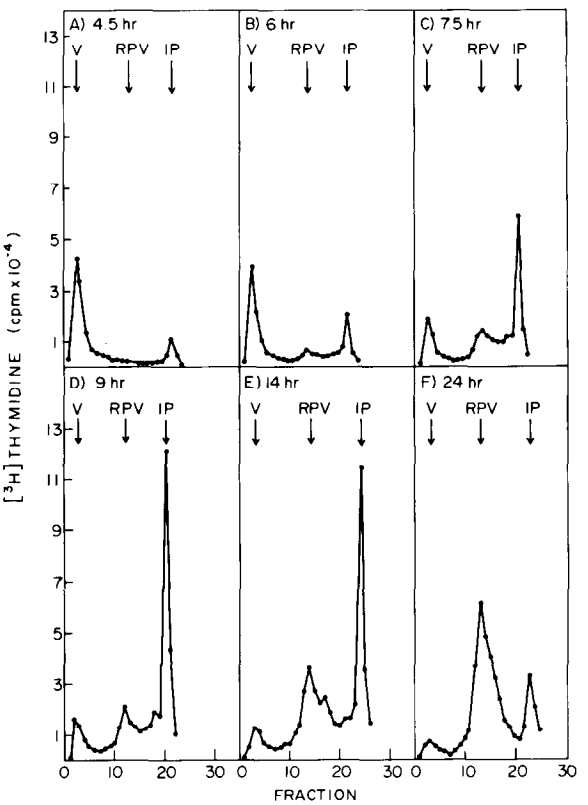

Figure 2. Conversion of Virosomal RPV DNA into Immature Particles and Then into Virus

Dishes of PK cells (1 × 10^{7} cells per dish) were infected and continuously exposed to ^{3}H-thymidine (2 μCi/ml) beginning 2 hr after infection. Cytoplasmic extracts were prepared from individual dishes harvested at the times indicated in the figure and subjected to sedimentation on sucrose gradients as described in Figure 1 and Experimental Procedures. Sedimentation positions of virosomes (V), mature rabbit poxvirus (RPV) and immature particles (IP) are shown. The direction of sedimentation is from right to left.

other investigators (Joklik and Becker, 1964; Dahl and Kates, 1970) suggest that at about 5 hr after infection morphogenesis begins and the DNA is rapidly excised from the pool of (previously stable) virosomes and assembled into slowly sedimenting immature virus particles. We would then predict these immature particles will, with time, undergo further differentiation into mature virus. This predicted pattern of DNA flow has been demonstrated by following the compartmentalization of continuously labeled progeny viral DNA as it is synthesized and matures within the infected cell (Figure 2). The viral DNA synthesized from 2 to 4.5 hr after infection first accumulates in virosomes (Figure 2A), in agreement with the data of Figure 1A. At this time, virosomes remain metabolically quite stable, as they are not yet utilized for particle formation. Between 6 and 9 hr after infection, even though the total radioactivity incorporated into viral DNA increases, the amount of material in virosomes decreases as immature particle formation begins and the virosomal lifetime continually shortens. As a consequence of the virosomal breakdown, the counts in immature particles increase sharply (Figures 2B, 2C and 2D), suggesting that this is the first stable product of maturation. We first detect material that sediments as virus 7.5 hr after infection (Figure 2C), but, compared to the rate of immature particle formation from virosomes, the conversion of immature particles to mature virus is very slow. The virus yield increases slowly from 7.5 hr, when it is first detected, to 24 hr after infection (Figures 2C–2F) at the expense of material in the immature particles. At 9 hr after infection, no further viral DNA is synthesized, so that thereafter (to 24 hr) one observes only the simple conversion of immature particles to virus. Several points can be made from the data of Figure 2. First, the flow of viral DNA following its synthesis is from virosomes into immature particles and thereafter into virus. Second, these immature particles serve as productive intermediates during viral morphogenesis, and, finally, the utilization of DNA contained within virosomes to form immature particles is rapid and the rate-limiting step in viral morphogenesis is differentiation of immature particles into mature virus.

We used the simple sedimentation procedure described in Figures 1 and 2 to separate these various pools of DNA in order to compare the restriction enzyme patterns of DNA from both virosomes and immature particles. Our purpose was to gain insight into the nature of the intermediate forms of DNA that are involved in replication and to determine the mechanism by which virosomal DNA might be utilized as the source of DNA for packaging.

Restriction Enzyme Analysis of DNA Derived from RPV Virosomes and Immature Particles

The initial purpose of our work was to address the question of whether concatamer or circle formation occurs during RPV replication. The formation of either concatamers or circles would involve a joining of the terminal regions of the genome to create intermediates, which after restriction enzyme digestion would result in the appearance of nonparental DNA "fusion fragments." Circular intermediates would result in head-to-tail joining of the molecule as a consequence of the fusion of the left (L) and right (R) ends. Concatamer formation, however, could result from either head-to-tail (LR) or alternating head-to-head (LL) and tail-to-tail (RR) joining. Newly synthesized poxvirus DNA contains many nicked and gapped regions (Esteban and Holowczak, 1977; Pogo and O'Shea, 1978). A genome containing nicks and gaps complicates the isolation and subsequent restriction enzyme analysis of the DNA. We find that restriction fragments from newly labeled DNA that are larger than about 10 $\times$ 10^6 daltons are unstable and present in submolar amounts. For our studies, therefore, we chose the enzyme Xho I, which cuts parental RPV DNA within the inverted terminal repeat present at each end to generate a two molar 3 $\times$ 10^6 dalton cross-linked terminal Xho I J fragment. The use of this enzyme allows us to determine whether there is end-to-end joining, because unless a new cleavage site is generated as a consequence of fragment fusion, all the interactions described above would generate a common fusion fragment whose predicted molecular weight (6 $\times$ 10^6) would still be small enough to allow quantitative evaluation. Figure 3 shows a comparison of the Xho I patterns of the DNA from mature wild-type RPV, from virosomes and from immature particles. Analysis of fragments on 0.4% agarose gels, to afford maximum separation of the larger fragments, shows that a new, nonparental fragment of 6.2 $\times$ 10^6 daltons (indicated by the asterisk) is readily visible in virosomal DNA but not in virion DNA (Figure 3, lane A). This 6.2 $\times$ 10^6 dalton band is present in only trace amounts in immature particle DNA, possibly as the result of contamination from sheared virosomal DNA generated during the sample preparation steps prior to resolution of the two DNA pools on sucrose gradients. We also separated the digests on 1% agarose gels to afford maximum separation of the smaller fragments and, in particular, to examine the fate of the Xho I J fragments present at both termini of parental DNA. Figure 3, lane B, shows that virosomal DNA lacks virtually all Xho I J fragment DNA, although this fragment is present in normal amounts in both parental and immature particle DNA. The presence of a new fragment of 6.2 $\times$ 10^6 daltons, together with the disappearance of the terminal parental Xho I J fragments in virosomal DNA strongly suggest that either circles or concatamers are formed during RPV replication. Furthermore, the data of Figures 1 and 2 suggest that the circular or concatameric virosomal DNA serves as the source of immature particle DNA in which the parental-like termini have been regenerated.

For the reasons outlined above, the choice of the

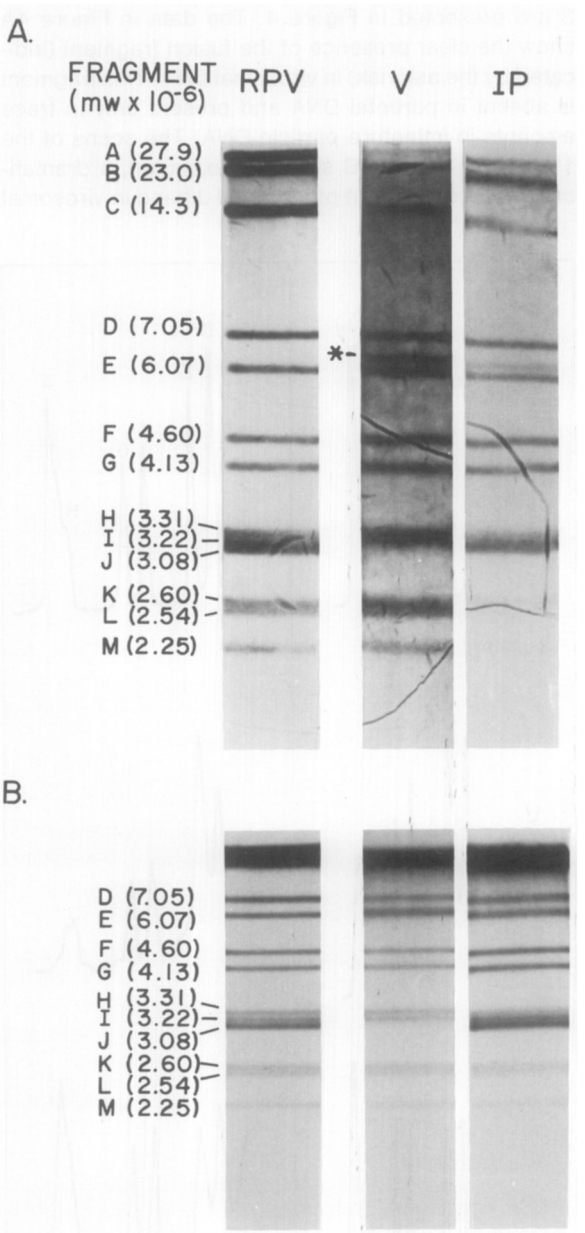

Figure 3. The Electrophoretic Analysis of Xho I Restriction Fragments Generated from the DNA of Virosomes and Immature Particles in RPV-Infected Cells

Samples of ^{3}H-DNA from purified wild-type rabbit poxvirus (RPV) as well as from virosomes (V) and immature particles (IP) isolated from RPV-infected cells were digested with Xho I. The resulting fragments were separated on 0.4% (lane A) and 1% (lane B) agarose gels and visualized by fluorography. The proposed nonparental fusion fragment appearing in virosomal DNA is indicated by an asterisk.

enzyme Xho I does not distinguish between head-to-tail versus head-to-head and tail-to-tail joining in concatamers. Densitometric scans of the gels, however, do permit an estimation of the number of termini that are joined and, if the molecules are concatameric rather than circular, an estimation of the average concatamer length. Scans of the gels shown in Figure 3 are presented in Figure 4. The data in Figure 4A show the clear presence of the fusion fragment (indicated by the asterisk) in virosomal DNA. This fragment is absent in parental DNA and present only in trace amounts in immature particle DNA. The scans of the 1% gels in Figure 4B show, as expected, a dramatically reduced amount of the Xho I J band in virosomal DNA as compared with either RPV or immature particle DNA. By mass, 50% of the incompletely resolved Xho I HIJ peak of parental virus DNA should be derived from the two molar terminal Xho I J fragment. We conservatively estimate that 75% of the Xho I J fragment of virosomal DNA is now found as the fusion fragment. If the termini were fused to create only concatamers, the average size of these concatamers would be four genomes in length.

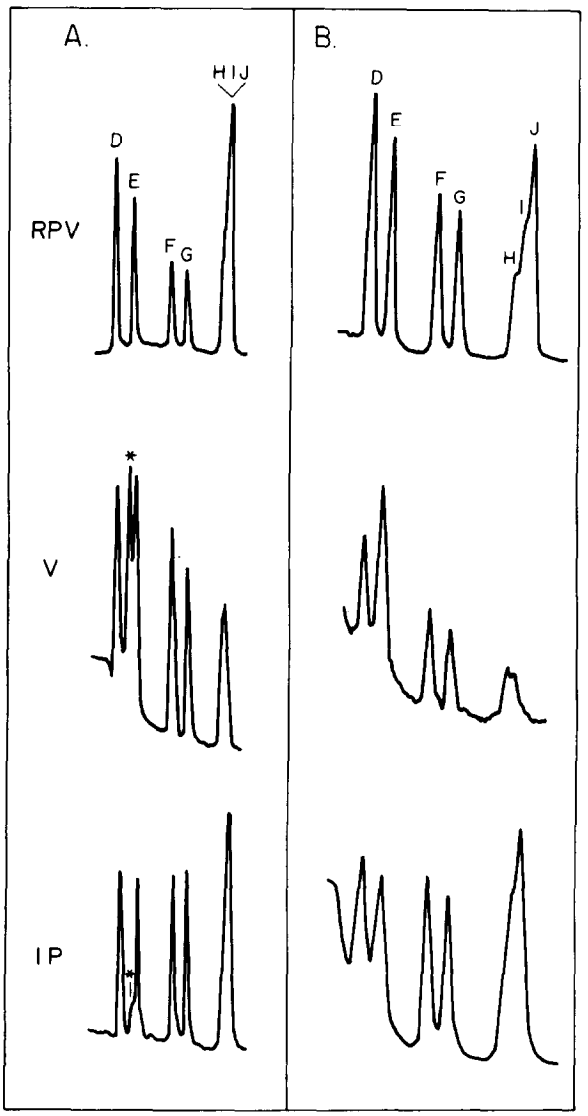

Figure 4. Quantitative Evaluation of the Gels of Figure 3

Scans of the 0.4% (A) and 1% (B) gels in Figure 3 are presented. The nonparental fusion fragment in virosomal (V) DNA migrating between the parental Xho I D and E fragments is identified with an asterisk. Electrophoresis is from left to right.

Virosomal DNA Consists of Linear Concatameric Molecules Joined in Alternating Head-to-Head and Tail-to-Tail Linkage

Given the technical necessity of analyzing restriction fragments of fairly low molecular weight in replicating DNA, we have addressed the specific nature of termini interaction through the use of a white pock (μ), host range (hr) mutant of RPV designated RPμhr28. This mutant does not yield mature virus in the pig kidney cell line used in these studies (Moyer and Rothe, 1980); however, DNA replication occurs, and normal maturation begins but is aborted after the formation of an immature particle (R. W. Moyer and R. L. Graves, manuscript in preparation). We have previously shown by restriction enzyme analysis that RPμhr28 is a deletion mutant of RPV (Moyer and Rothe, 1980). For this study, the relevant feature of this mutant is that, because of the deletion, Xho I digestion of mature viral RPμhr28 DNA generates two easily separated cross-linked terminal fragments with molecular weights of 4.8×10^6 for the left (L) terminal fragments and 3.08×10^6 for the right (R) terminal fragment. Head-to-tail (LR) joining of the terminal fragments within virosomes, whether through circles or linear concatamers, would yield a single fusion fragment of approximately 8×10^6 daltons. Conversely, if molecules are joined through alternating head-to-head (LL) and tail-to-tail (RR) linkage, we would expect virosomal DNA to contain two new fusion fragments with molecular weights of approximately 9.6×10^6 if derived from the sum of two left (LL) terminal fragments or 6.2×10^6 if derived from the sum of two right (RR) terminal fragments. These two fusion fragments would be in equal molar amounts relative to each other, but would be in no more than a half molar ratio to the remaining unaffected DNA fragments of the genome.

The data in Figures 5 and 6 show the Xho I fragments of parental RPμhr28 DNA compared with two preparations of RPμhr28 virosomal DNA. As observed above for wild-type RPV virosomal DNA, both preparations of RPμhr28 virosomal DNA lack any significant quantity (less than a 0.25 molar ratio) of either the left or right parental terminal DNA fragment (Figure 5, lanes B and C). Instead, three new nonparental bands appear with molecular weights that correspond to the expected sizes of fusion fragments resulting from the joining of two left ends (LL, $\sim 10 \times 10^6$), one left and one right end (LR, $\sim 8 \times 10^6$), and two right ends (RR,

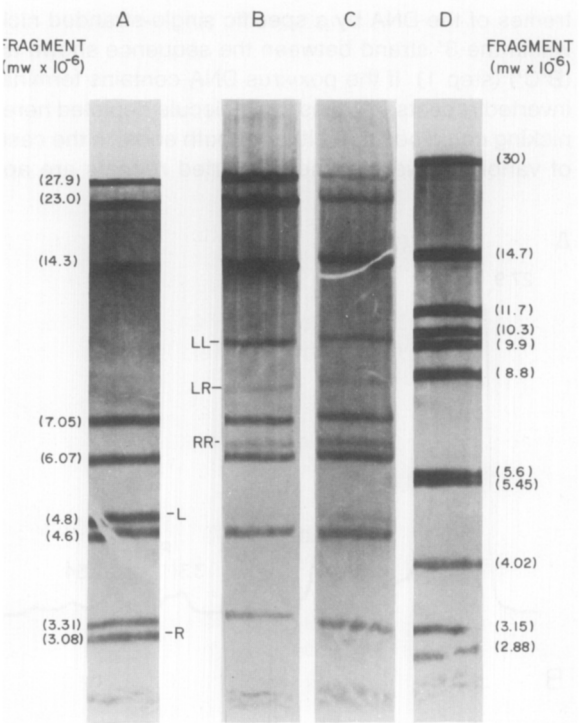

Figure 5. Electrophoretic Analysis of RPμhr28 Virosomal DNA Fragments Generated by Digestion with Xho I

Samples of RPμhr28 viral DNA digested with Xho I (lane A), two independent preparations of intracellular RPμhr28 virosomal DNA digested with Xho I (lanes B and C) and RPμhr28 viral DNA digested with Hind III to provide molecular weight markers (lane D) were separated by electrophoresis on 0.4% agarose gels and visualized by fluorography. The left (L) and right (R) terminal Xho I fragments of parental RPμhr28 DNA are shown in (lane A). The nonparental DNA Xho I fragments created by the fusion of two left (LL), two right (RR) and the left and right (LR) ends present in the two samples of virosomal DNA (lanes B and C) are indicated only in (lane B).

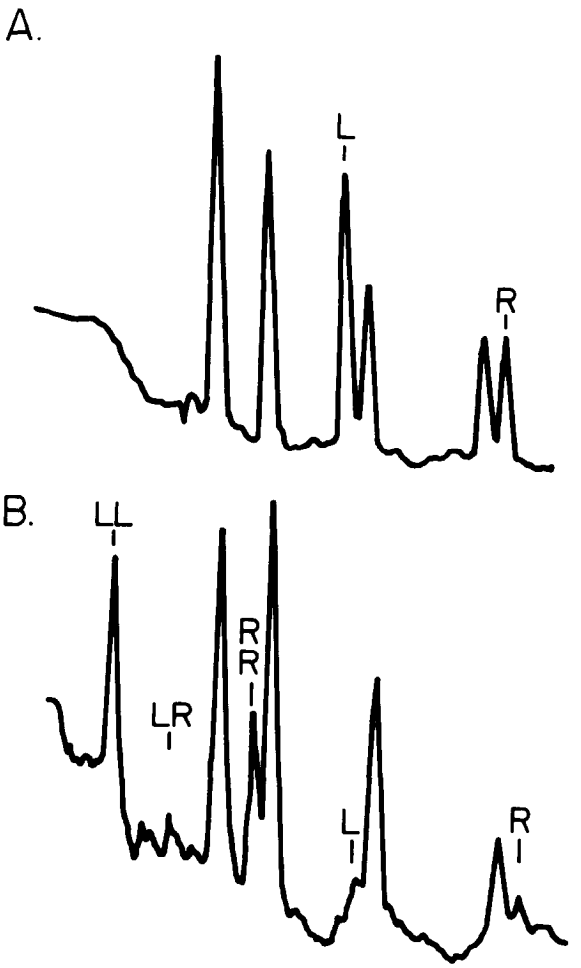

Figure 6. Densitometric Quantitation of the Gels of Figure 5

Densitometric quantitation of the separated Xho I fragments of parental RPμhr28 (A) and of RPμhr28 intracellular virosomal DNA (B, lane C) was performed on the autoradiograms depicted in Figure 5. Electrophoresis is from left to right.

~6.2 × 10^6). Densitometric scans of these gels (Figure 6) reveal that the majority of the fusion fragments are (LL) and (RR) in approximately equal molar ratios to each other with only a small percentage (5%) being of the (LR) variety.

More direct evidence that the proposed (LL) and (RR) fusion fragments of virosomal DNA are derived from the terminal fragments of RPμhr28 DNA can be provided by the ability of these fragments isolated from gels to hybridize to the (L) and (R) terminal fragments of RPμhr28 DNA. Attempts to obtain pure (LL) and (RR) fragments from gels for such hybridization experiments are hindered by the inherent heterogeneous nature of virosomal DNA discussed above, which results in a high background of radioactive material throughout the separating gel (see Figure 5). Both isolated fusion fragments therefore suffer contamination with breakdown products of higher molecular weight fragments, a problem that is particularly acute in the case of the larger (LL) fragment. In spite of this difficulty, we have compared the ability of the ^{32}P-labeled (LL) and (RR) fusion fragments, as well as total ^{32}P-labeled virosomal DNA, to hybridize to blots of Xho I–digested RPμhr28 viral DNA (Figure 7). Total virosomal DNA hybridizes increasingly to fragments of higher molecular weight, yielding equimolar hybridization to all fragments as expected (Figure 7A). The hybridization of the (LL) fusion fragment, unlike total virosomal DNA, yields relatively strong signals from both the (L) and (R) terminal fragments of RPμhr28 DNA (7B). This result is expected since a portion of the (L) terminal fragment consists of the inverted terminal repeat sequences common to both the (L) and (R) terminal fragments. Significant hybridization of contaminating material in the partially pure (LL) fragment is observed to the four parental fragments of higher molecular weight (the doublet at 14.3 × 10^6, 23 × 10^6, and 27.7 × 10^6). However, the relative increase in hybridization expected for the (LL) fusion fragment to the (L) and (R) terminal fragments of parental DNA as compared with that observed for total virosomal DNA (Figure 7A) is

obvious. The hybridization of the purified (RR) fusion fragment to blots of parental RPμhr28 DNA is shown in Figure 7C. The (RR) fusion fragment, unlike total virosomal DNA, hybridizes to a high degree equally well with both the (L) and (R) terminal fragments of parental DNA. This result is expected since the (RR) fusion fragment consists solely of inverted terminal repeat sequences shared by both the (L) and (R) terminal fragments. The presence of some contaminating DNA material is evident through hybridization of radioactivity to other fragments of higher molecular weight. As we noted for the hybridization of the (LL) fragment, however, the significant increase in the relative hybridization of the isolated (RR) fragment to the (L) and (R) terminal fragments of parental RPμhr28 DNA as compared with that of the total virosomal DNA (Figure 7A) is obvious.

The observed sizes of the nonparental (LL) and (RR) Xho I fusion fragments in virosomal DNA shown in Figure 5, together with the hybridization data of Figure 7, leave little doubt that the (LL) and (RR) fragments originate as a result of head-to-head and tail-to-tail arrangement of genomes within concatamers. Since most (95%) of these fusion fragments are the (LL) and (RR) varieties, we conclude that newly replicated virosomal DNA consists of linear concatamers, on the average of four genomes in length, joined in alternating head-to-head and tail-to-tail arrays. It is also this concatameric DNA from which the unit length DNA of immature particles is derived. A corollary of these conclusions it that any circles or concatamers resulting from the small percentage of head-to-tail (LR) fusions are probably not obligatory intermediates in the replicative process of poxvirus DNA.

A Model for the Replication of Orthopoxvirus DNA

The detection of DNA concatamers composed of genome length DNA joined in alternating head-to-head and tail-to-tail linkage leads us to propose the replication scheme shown in Figure 8. This scheme is similar to that proposed for the parvoviruses (Tattersall and Ward, 1976; Straus et al., 1976), but differs significantly from the model for poxvirus DNA replication proposed by Esteban et al., 1977. The cross-linked RPV parental DNA molecule is shown in Figure 8, with the overall directional orientation of the complementary sequences within the left end, indicated by (L/L′), and within the right end, indicated by (R/R′). Following the left-hand single-stranded cross-link, sets of genes beginning at the leftmost extreme of the double-stranded DNA are represented with the letters A/A′ to D/D′ and ending in Z/Z′ at the right end. An inverted terminal repeat following the Z gene at the right end of the genome is indicated by (C′B′A′/CBA). The inverted repeat is then followed by the right-hand single-stranded cross-link.

We propose that replication is initiated within the double-stranded region near one of the terminal extremes of the DNA by a specific single-stranded nick within the 3′ strand between the sequence shown as (B′C′) (step 1). If the poxvirus DNA contains terminal inverted repeats, as does the molecule depicted here, nicking could occur at either or both ends. In the case of variola, however, where inverted repeats are ap-

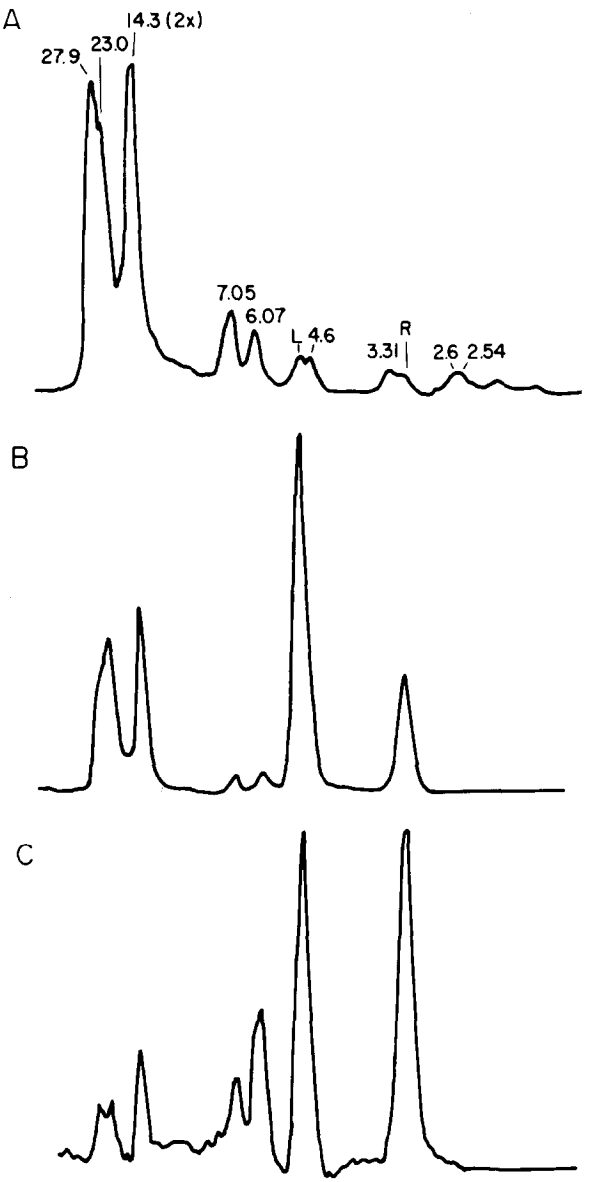

Figure 7. Hybridization of Purified ^{32}P-Labeled (LL) and (RR) Xho I Fusion Fragments from Concatameric RPμhr28 DNA to Immobilized Xho I Fragments of RPμhr28 Virion DNA

The conditions for the preparation of ^{32}P-labeled RPμhr28 virosomal DNA for Xho I digestion and subsequent fragment separation on agarose gels is described in Experimental Procedures. Procedures for the isolation of the appropriate fusion fragments from the agarose gels and conditions for the hybridization of ^{32}P-labeled DNA to unlabeled viral DNA fragments immobilized on nitrocellulose have been described (Moyer et al., 1980b; Ficht and Moyer, 1980). Separate hybridizations were performed with total virosomal DNA (A), purified LL fusion fragment (B) and purified RR fusion fragment (C).

parently absent, nicking might be limited to one end of the molecule. In our model, for simplicity, we initiate replication with only a single nick. In step 2, we have redrawn the parental molecule to illustrate that the (B′C′) nick (indicated by the arrow) has occurred on the 3′ strand near the left end of the molecule. We propose that the 3′ end can then be extended using the displaced complementary strand as template (step 3). This would first allow the copying of the sequences

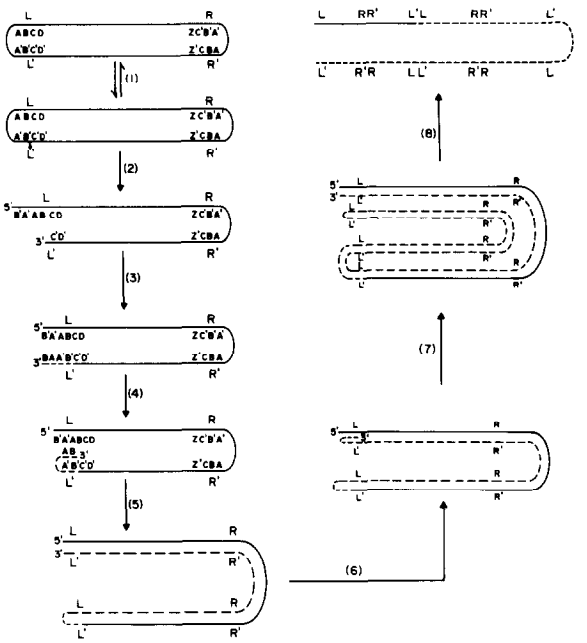

Figure 8. Model for the Replication of Orthopoxvirus DNA

Replication is initiated by the introduction of a nick into the 3′ strand at the (B′C′) sequence within the duplex region near the left end of the DNA. Parental DNA strands are indicated by solid (——) lines and newly synthesized progeny DNA strands with dashed (-----) lines. When inverted repeats are present (RPV, vaccinia), nicking can occur at both ends of the genome, allowing simultaneous replication from both ends and resulting in the synthesis of two unit-length, double-stranded parental-like molecules (such as those shown resulting from step 3 in the figure) as products of the first round of replication. See text for further details of the model.

constituting the cross-link and then create an inverted repeat (B′A′AB/BAA′B′) that could subsequently generate a priming hairpin structure to allow for the replication of the DNA (step 4). Strand extension would yield a complete copy of the genome and result in a dimeric molecule joined through two right (RR) ends in a tail-to-tail configuration (step 5). The process can be repeated (steps 6 and 7) to generate a tetrameric concatamer where the molecules are linked through two right (RR), then two left (LL) and finally two right (RR) terminal fragments.

If we redraw the final replication product, which can be viewed as a double-stranded concatameric hairpin (step 8) to show more clearly the alignment of the genomes, one would predict that restriction enzyme analysis should yield more fusion products derived from the right end than from the left end. In our studies with rabbit poxvirus, however, we found approximately equal molar amounts of (LL) and (RR) joined ends. This suggests either that the arrays are larger than a tetramer or, if the limiting size of the concatamer is a tetramer, that equal numbers of molecules must initiate replication at both ends since rabbit poxvirus contains inverted repeat sequences. Several features of poxvirus DNA replication become evident with the generation of this type of concatameric replicative intermediate: simple circular molecules formed from head-to-tail fusion are eliminated as obligatory molecular intermediates in DNA replication; the viral genomes within concatamers are aligned in mirror image arrays with respect to one another; and at no time is it necessary for the left and right ends of the molecule to interact directly.

The Excision of Genome-Length Molecules from Head-to-Head and Tail-to-Tail Concatameric Arrays during Immature Particle Formation

Previous results of other investigators (Joklik and Becker, 1964; Dahl and Kates, 1970) and the data of Figures 1 and 2 indicate that the DNA of virosomes is the substrate from which the mature-length genomes

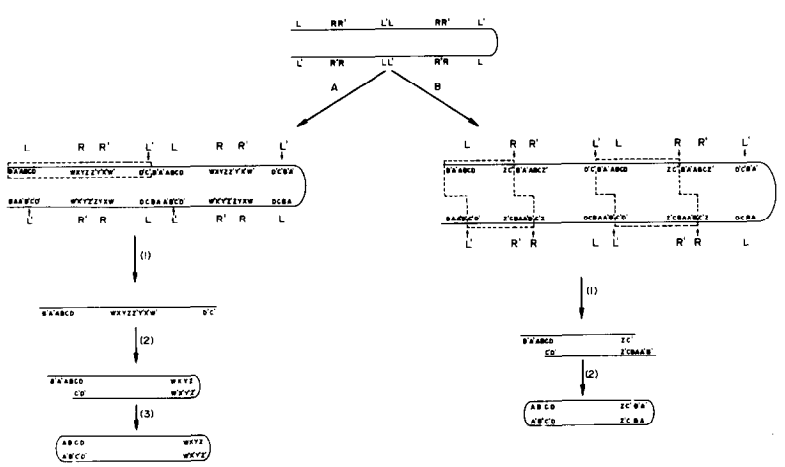

Figure 9. Excision of the Monomeric Molecules from Virosomal DNA Concatamers for Packaging

A mechanism is presented for the excision of unit length molecules from concatamers where molecular complexity is determined by a recognition sequence (B′C′). Poxviruses are considered that either lack inverted terminal repeats (scheme A) or contain inverted terminal repeats (scheme B). See text for further details.

of immature particles are derived. We propose in Figure 9 a mechanism for how DNA excision might occur. Any packaging scheme must consider not only the nature of the precursor concatameric DNA, but also that under certain conditions the genome size of a poxvirus can vary enormously. The DNA of wild-type RPV is noncircularly permuted and has a molecular weight of 118×10^6. Data from this laboratory have shown that under certain conditions infectious variants of RPV can be isolated that have molecular weights ranging from 98×10^6 (Moyer and Rothe, 1980) to 140×10^6 (Moyer et al., 1980b). These observations suggest that the size of the packaged genome is determined primarily by sequence recognition. We therefore propose that packaging is initiated by single strand nicking at a precise sequence within the concatamer (indicated arbitrarily in Figure 9 by arrows as between the [B'C'] sequences) where the length of the molecule to be packaged would depend on where the sequence is repeated. We begin with a tetrameric concatamer and consider the maturation of virus under two sets of conditions; that is, where the virus contains inverted repeats (vaccinia, rabbit poxvirus) and where the virus does not contain inverted repeats (variola). In the case of orthopoxviruses lacking inverted repeats, we represent the complete double-stranded complementary sequence in the virus by the letters (A/A' to Z/Z'). A concatamer generated by this type of virus is shown in scheme A of Figure 9. The repeat distance of the sequence (B'C') determines the size of the DNA to be packaged (step 1). The DNA is then excised, annealed (steps 2 and 3), and ligated to yield a cross-linked mature genome. Although the concatamer is drawn in Figure 9 as a simple hairpin molecule four genomes in length, the alternating head-to-head and tail-to-tail alignment of genomes also allows for the formation of cruciform structures through self-annealing of the individual strands. Although we recognize that a cruciform arrangement of the concatamer might be the substrate utilized in the packaging process, in the interest of simplicity we have not indicated these structural alternatives. In scheme B of Figure 9, we show a proposed maturation scheme for a poxvirus containing an inverted terminal repeat. As in Figure 8, the genes of the virus include the sequences (A/A') to (Z/Z'), followed by the inverted repeat (C'B'A'/CBA). Packaging is dictated by the spacing of the (B'C') nicking site (indicated by the arrows) as shown in step 1. Mature viral DNA forms following self-annealing (step 2) and ligation.

A Proposed Mechanism for the Generation of Poxvirus Variants Containing Either Terminal, Mirror Image Gene Duplications or Deletions

One of the most remarkable classes of poxvirus variants is that which contains large mirror image duplications of either the left or right regions of the genome. The scope and variability of these mutants are illustrated by some of the RPV variants we have mapped (Figure 10). All these mutants were originally isolated as white pock (μ) variants, although a number of them also show host range (hr) properties. Duplications of both left-hand sequences (RPμ21, RPμ7, RPμ26) and right-hand sequences (RPμ81g, RPμhr125, RPμhr30) of parental RPV DNA are equally common. Up to 25% of the parental right-hand terminal sequences (RPμ81g) and 22% of the parental left-hand terminal sequences (RPμ26) can be duplicated and stably maintained in variants as large, newly created inverted repeats. Every mutant of this type that we have examined also contains a variable deletion of the original parental sequences in the terminus modified by the addition of genes from the opposite end of the genome.

We propose that gene duplication mutants of the type described in Figure 10 could arise by internal deletions within the head-to-head and tail-to-tail concatamers prior to packaging, as illustrated in Figure 11. One can consider the viral concatamer as either variola-like—that is, the left and right ends are unique—or RPV-like with terminal repeats. Following

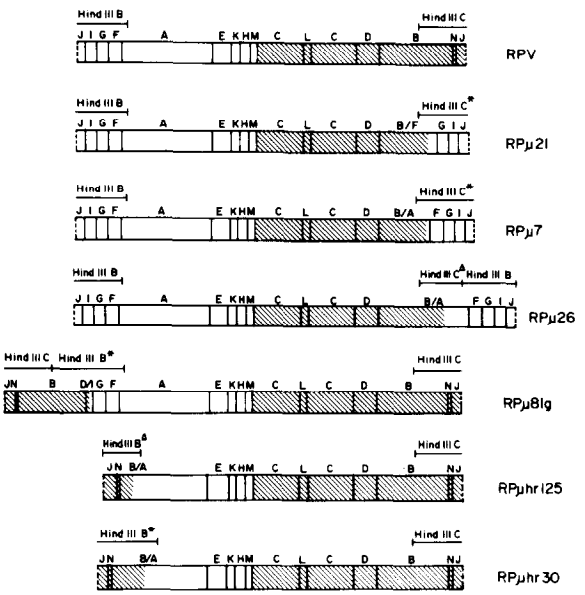

Figure 10. Genome Variability of Rabbit Poxvirus

The Xho I maps of parental RPV and six variants are presented. The left and right halves of the parental RPV genome are arbitrarily distinguished by crosshatching to demonstrate more readily the original source of the terminal sequences transposed in the variants. Maps both of white pock (μ) mutants and of white pock mutants with a reduced host range (μhr) are shown. The terminal fragments resulting from Hind III digestion are also shown. Mutant RPμ81g has the unimolar cross-linked Hind III C fragment of RPV in two molar amounts. Similarly, RPμ26 has the unimolar cross-linked Hind III B fragment of RPV present in two molar amounts. The maps are drawn to scale to emphasize the net insertions of DNA in mutants RPμ21, RPμ7, RPμ26 and RPμ81g and the net deletions of DNA in mutants RPμhr125 and RPμhr30. Parental Hind III fragments in mutants that contain deletions (Δ) or insertions (*) are also indicated.

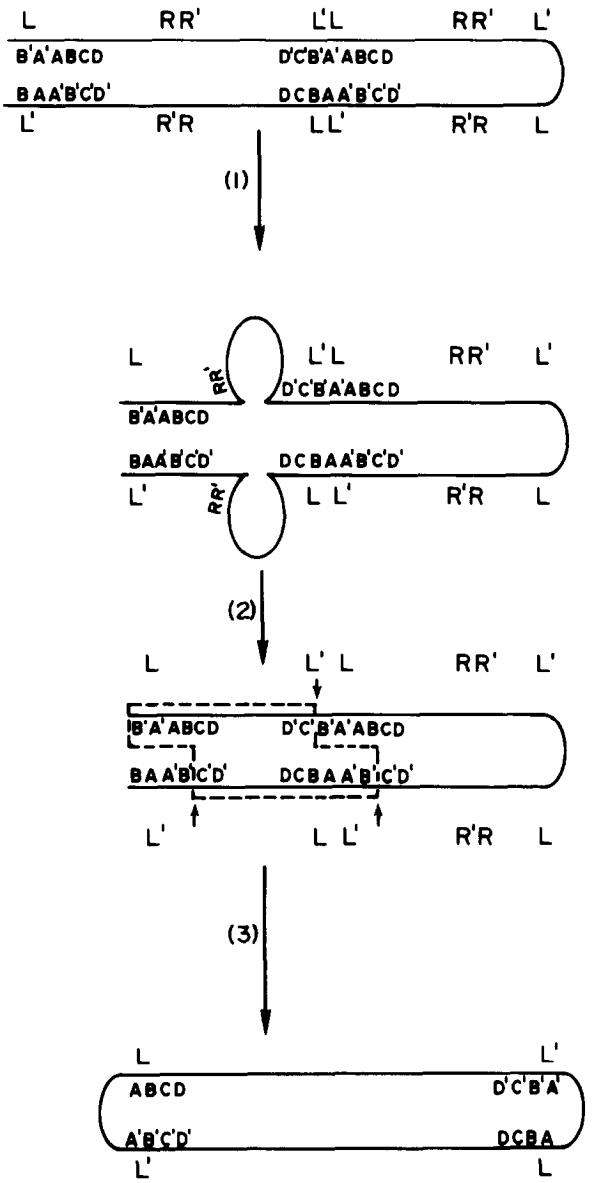

Figure 11. Generation of RPV Gene Duplications and Inverted Repeats

A concatameric RPV DNA array is depicted as in Figure 9. Following deletion (step 1), molecular excision governed by the spacing of the (B'C') recognition sequence occurs and, in the example illustrated, leads to a duplication of left-hand sequences and the creation of an expanded inverted terminal repeat.

deletion (step 1), packaging is initiated by nicking and excision at the (B'C') recognition site (step 2). The ultimate size and sequences to be packaged are determined by the spacing of the (B'C') recognition sequence. Upon reannealing and ligation (step 3), a viral variant is generated that contains duplications of left-hand genes present as mirror image inverted repeats. Mirror image deletions can also easily arise by the same mechanism. If the starting concatamer is derived from a variola-like virus, inverted terminal repeats will be generated in a variant from a parental virus in which they did not exist. RPV variants containing these large inverted repeats are generated at a significant rate by passage of the virus in eggs. We suggest that deletions within the replicative intermediates and a recombination mechanism proposed earlier (Moyer et al., 1980b) could together account for the high frequency of variant formation.

Given that virosomal DNA consists of genomes aligned in mirror image arrays, the existence of mutants with variable molecular weights as shown in Figure 10 offers a compelling argument in favor of a genome size determined by a sequence specificity that governs molecular excision. It has been shown that the DNA contained within the inverted terminal repeats is transcribed and translated (Wittek et al., 1980). Although the genes of the inverted terminal repeat may be essential for growth, one copy may be sufficient and the inverted terminal repeats per se may not be an essential feature of the virus. For instance, the human variola virus is the most fastidious member of the orthopoxvirus family with respect to permissible hosts and growth conditions, yet this virus has no inverted terminal repeats characteristic of the laboratory strains of vaccinia and RPV, which are grown and maintained in cell culture (Dumbell and Archard, 1980). As we pointed out in the discussion of the RPV variants depicted in Figure 10, each duplication of genes at one terminus with their mirror image insertion into the opposite terminus is always accompanied by deletion in the terminus to be modified. The result is the generation of a variant with an inverted terminal repeat of sequences from one terminus with deletion of some original parental sequences. The possibility exists, therefore, that the inverted terminal repeats in laboratory strains are generated from a variola-like virus originally lacking inverted repeats as a consequence of viral propagation outside of the natural host, under conditions where some terminally located viral genes indispensable for propagation of the virus in the natural host can be eliminated. The formation of host range mutants of RPV (Moyer and Rothe, 1980), characterized by deletions of quasi-essential genes in the left-hand region of the genome, and of mutants with greatly expanded terminal repeats (Moyer et al., 1980b) are both consistent with this view.

Experimental Procedures

Cells and Virus

Wild-type rabbit poxvirus (RPV, Utrecht strain) and pig kidney (PK) cells were obtained from the American Type Culture Collection. The white pock (μ), host range (hr) mutant RPμhr28 has been previously described (Fenner and Sambrook, 1966; Moyer and Rothe, 1980). The routine propagation of virus was on chicken embryo fibroblasts (CEF) as described (Moyer and Rothe, 1980). PK cells were maintained in Eagle's essential medium (Gibco F-11) supplemented to contain 5% fetal bovine serum, 5% porcine serum, 2 mM glutamine, 100 units penicillin, 100 μg streptomycin and 0.1 mg/ml sodium pyruvate.

Preparation of ^{3}H-Labeled Viral DNA

^{3}H-labeled RPV and RPμhr28 viral DNA were prepared by infection of chicken embryo fibroblasts (2×10^8 cells) at a multiplicity of infection (moi) of 0.1 in CEF medium containing dialyzed serum but lacking tryptose phosphate broth (Moyer and Rothe, 1980). The infected cells were incubated for 72 hr in the presence of 5 μCi/ml ^{3}H-thymidine (50–80 Ci/mmole) before harvest of the virus and purification of the DNA (Moyer and Rothe, 1980).

Preparation of ^{3}H-Labeled Intracellular RPV and RPμhr28 DNA

PK cells were infected at moi 10, in medium that contained dialyzed serum as described by Moyer et al. (1980a). Following an adsorption period of 2 hr, ^{3}H-thymidine (50–80 Ci/mmole) was added as described for individual experiments. When cytoplasmic compartmentalization of newly labeled RPV DNA was to be measured, infected cells (1×10^7 total cells) were exposed to 2 μCi/ml ^{3}H-thymidine for periods beginning at the times after infection indicated in individual figures. When ^{3}H-labeled RPV DNA was to be prepared for restriction enzyme analysis, infected PK cells (4×10^7 total cells) were exposed to 50 μCi/ml ^{3}H-thymidine from 2.5 to 3.25 hr after infection to label RPV virosomal DNA and from 5 to 6 hr after infection to label RPV immature particle DNA. Virosomal RPμhr28 DNA was labeled from 2 to 4.75 hr after infection.

Preparation of ^{32}P-Labeled RPμhr28 Virosomal DNA

Slightly subconfluent monolayers (8×10^7 total cells) were starved for phosphate and infected with RPμhr28 as described by Moyer et al. (1980b). The cells were labeled with ^{32}P-orthophosphate (66 μCi/ml) from the time of infection until 4.75 hr after infection. After scraping the cells from dishes for harvest, we combined them with 4×10^7 unlabeled cells infected with RPμhr28 for 4.75 hr before isolating the virosomal DNA.

Preparation of Cytoplasmic Extracts and Fractionation of Newly Synthesized Cytoplasmic Viral DNA

The cells were scraped into the medium and collected by centrifugation at 800 × g for 5 min. All further operations were at 4°C. The cells were suspended twice in 5 ml of a solution that contained 0.15 M NaCl, 5 mM EDTA and 0.05 M Tris–HCl (pH 8.0) and collected as before. The cells were resuspended at a concentration not exceeding 5×10^7 cells/ml in lysing solution (0.01 M KCl, 5 mM EDTA, 0.01 M Tris–HCl [pH 8.0]), homogenized with ten strokes of a Dounce homogenizer and incubated for 5 min. The cells were then treated with Triton X-100 at a final concentration of 0.1% for 5 min. The nuclei were removed by centrifugation at 800 × g for 3 min. The fractionation of newly synthesized cytoplasmic viral DNA was achieved by centrifugation of the cytoplasmic extracts on linear, 38% to 50% (w/v) sucrose gradients prepared in SW27 centrifuge tubes atop a 5 ml cushion of 84% (w/v) sucrose by a procedure similar to that of Dahl and Kates (1970). All sucrose solutions were prepared in 0.05 M NaCl, 1 mM EDTA and 0.01 M Tris–HCl (pH 8.0). Centrifugation was for 18,000 rpm for 30 min at 4°C in the Beckman SW27 rotor. Under these conditions, virosomes sediment to the bottom of the gradient atop the 5 ml cushion. Gradients were collected from the bottom with the aid of a peristaltic pump.

Purification of Virosomal and Immature Particle DNA

Sucrose gradient fractions of virosomal and immature particle DNA were adjusted to contain 1% sodium deoxycholate and 1 mg/ml Pronase (self-digested for 30 min at 37°C) and were incubated overnight at 37°C. The samples were deproteinized by phenol-extraction as described previously (Moyer and Rothe, 1980).

Restriction Enzyme Digestion of DNA and Analysis of Fragments by Agarose Gel Electrophoresis

All radiolabeled DNA samples were supplemented with 10 μg of unlabeled DNA from the parental virus before digestion with Xho I. The digestion conditions, general electrophoretic procedures and conditions for fragment isolation from gels and the subsequent hybridization of the purified DNA to viral DNA immobilized on nitrocellulose strips have been described (Moyer et al., 1980b; Moyer and Rothe, 1980). The electrophoresis of samples in 0.4% gels (20 cm in length) was for 24 hr at 36 V, whereas samples in 1% gels were subjected to electrophoresis for 21 hr at 28 V. Agarose gels were prepared for fluorography by two 30 min washes in 95% ethanol, followed by a 3 hr wash in 5% PPO in acetone. Gels were rehydrated in water before drying and fluorography (Bonner and Laskey, 1974). Densitometric measurements were made with the use of a Zeineh soft laser densitometer. The linearity of the fluorograms was ensured by the inclusion of uniformly labeled ^{3}H-viral DNA standards in all experiments.

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