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Biogenesis of Poxviruses: Mirror-Image Deletions in Vaccinia Virus DNA

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Summary

Restriction endonuclease analysis of viral DNA extracted from wild-type and temperature-sensitive mutants of vaccinia IHD-W (Dales et al., 1978) revealed sequence alterations in approximately 20% of all *ts* clones examined. The rearrangements were due to deletions up to 250 nucleotide pairs long. Using Eco RI, Sal I, Bam I, Hpa I and Ava I, the deletions were always observed in the same fragments, while analysis with Hind III demonstrated deletions of identical size in the two terminal fragments. Since vaccinia virus contains inverted terminal repeats of more than 10 kb, these clones possess identical deletions of opposite orientation at both ends of the genome. Analysis of several revertants of the *ts* mutants demonstrated that the deletions probably arise as events independent from those producing *ts* lesions and are generated spontaneously at high frequency. This implies that a single event during replication caused the elimination of nonessential information, and suggests that circular intermediates must exist transiently during viral replication.

Introduction

Poxviruses are probably the most complex of all animal agents and possess linear DNA genomes of >120 $\times 10^6$ daltons (Müller et al., 1977; Esposito, Obijeski and Nakano, 1978; McCarron et al., 1978) which can potentially encode for over 200 hundred proteins, more than 100 being present in the virion itself (Essani and Dales, 1979). This complexity is to some degree reflected by the nature of virus replication within cytoplasmic "factories," which in large measure is autonomous from the activities of the host nucleus.

In the course of our investigations concerning the biogenesis of vaccinia virus, we have accumulated approximately 90 conditional lethal or temperature-sensitive (*ts*) mutants with defects at different stages of the poxvirus life cycle (Dales et al., 1978). Since complementation and recombination data strongly suggested that the majority of these *ts* isolates expressed their mutant phenotype by virtue of single independent mutations (Lake, Silver and Dales, 1979), we initiated a routine screening of the various isolates for evidence of mutated sites in the viral DNA detectable by alterations in the recognition sequence for any of the commonly available restriction endonucleases.

During the course of our survey using restriction endonucleases, it was unexpectedly observed that a significant proportion of the vaccinia clones studied had developed unique deletions in the genome, some as extensive as 250 base pairs (bp). We report here that all such deletions were present within the inverted terminal repetitions of vaccinia DNA identified by Garon, Barbosa and Moss (1978) and Wittek et al. (1978a). Furthermore, the missing segments invariably occurred symmetrically at both ends of the genome, leading us to hypothesize that at some stage of replication, vaccinia DNA must undergo transient circularity. In the Discussion, we consider the possible relevance of these unique deletions to current models for the replication of linear DNA genomes, such as that of vaccinia virus.

Results

Analysis of Vaccinia Temperature-Sensitive Mutants by Restriction Endonucleases

During our investigations of the molecular basis for temperature sensitivity of the vaccinia mutants in our collection (Dales et al., 1978), we initiated a program of screening the isolates for alterations in the primary structure of the DNA. To expedite the purification of viral DNA from a large number of mutants, we used a modification of a lysis gradient technique (Parkhurst and Heidelberger, 1976) which allows complete separation of viral proteins from the nucleic acid in a single step. As shown in Figure 1, ^{14}C-labeled DNA sedimenting at 76S could be separated from both detectable ^{3}H viral protein and any contaminating host DNA. Viral DNA purified in this way was digested to completion by all restriction enzymes tested, and in control gels there was no evidence of detectable host background.

To date we have examined all the members of both the DNA$^-$ (in preparation) and the maturation-defective (Lake et al., 1979) vaccinia *ts* groups with 5–6 restriction endonucleases, and have observed only one isolate which has an altered restriction profile after electrophoresis in agarose gels. This particular mutant [designated *ts* 9251 by Dales et al. (1978)], which possesses an additional Eco R site in fragment D, will be extensively characterized in a subsequent communication.

Some of the Vaccinia *ts* Isolates Contain Small Deletions

When screening for single base pair alterations such as the one associated with *ts* 9251 was begun, it was expected that the total molecular weight of the viral DNA in the various mutants would be identical. When,

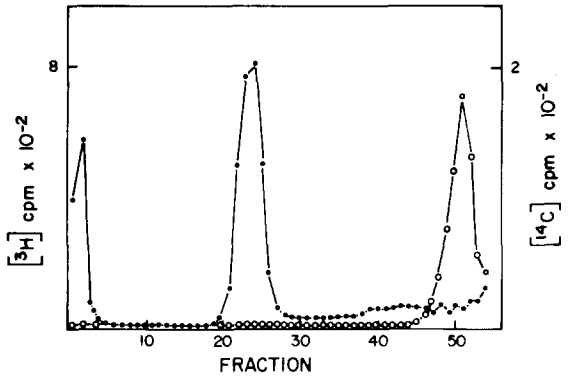

Figure 1. Purification of Vaccinia DNA by Centrifugation in Lysis Gradient

Vaccinia virions labeled with ^{3}H amino acids and ^{14}C-thymidine were purified and centrifuged in a neutral sucrose gradient containing sodium dodecylsulfate and β-mercaptoethanol, as described in Experimental Procedures. (○) ^{3}H; (●) ^{14}C.

however, the DNA from mutants belonging to various classes described by Dales et al. (1978) was digested with Eco RI and analyzed by electrophoresis in 0.7% agarose gels, a significant fraction was shown to differ from the parental size. This is clearly illustrated in Figure 2, in which cleavage profiles of one class of *ts* vaccinia mutants, defective in viral DNA synthesis, are illustrated. The channel marked IHD-W reveals the digestion pattern of wild-type vaccinia DNA exposed to Eco RI. This pattern is significantly different from those published on other serotypes (Müller et al. 1977; McCarron et al., 1978), but the cumulative molecular weight of approximately 120×10^6 daltons is similar. While *ts* 4149, *ts* 7790 and *ts* 8933 exhibited patterns identical to those of the prototype virus, *ts* 6389 and *ts* 8741 profiles possessed a restriction fragment of increased mobility in the zone ~3 $\times 10^6$ daltons. With *ts* 6389 and *ts* 8741, there was no evidence of any novel low molecular weight fragments to account for reduction in molecular weight of the one at $\sim 3 \times 10^6$ daltons. Digestion with Bam I, Hpa I and Sal I (Figure 3) revealed that in both *ts* 6389 and *ts* 8741, a single fragment of invariably reduced size occurred, indicating that both these mutants contained a deleted segment of DNA at approximately the same location in the viral genome. Similar changes in the mobility associated with fragments produced by Hind III digestion (Figure 3) could not be demonstrated by separation in these gels, because the larger fragments ($>10^7$ daltons) do not migrate strictly according to chain length in 0.7% agarose. However, the profile produced by Hind III was examined further, as described below, for comparative purposes, since it was the only endonuclease among those used which created a fragment profile of IHD-W vaccinia that was identical to those established for other vaccinia strains (Esposito et al., 1978; McCarron et al., 1978).

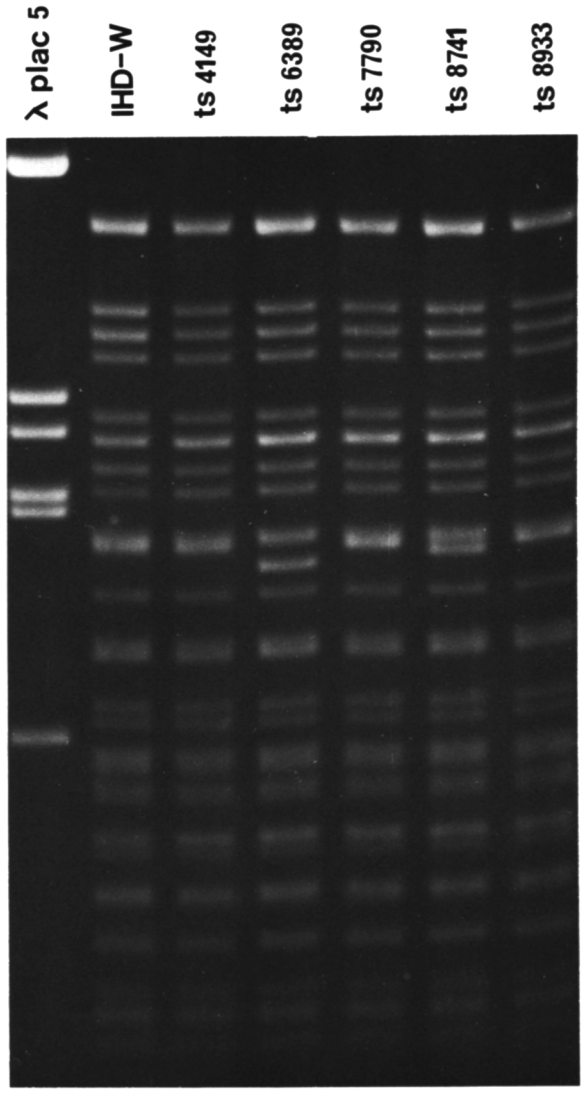

Figure 2. Analysis of Eco RI-Digested DNA from DNA$^-$ *ts* Mutants by Electrophoresis in 0.7% Agarose

Viral DNA from the various DNA$^-$ *ts* isolates was digested with Eco RI and analyzed by electrophoresis in 0.7% agarose. Eco RI-digested λ plac 5 was used as molecular weight marker (molecular weights = 12.4, 4.8, 4.6, 3.75, 3.5 and 2.1×10^6 daltons).

Size and Location of the Deletions

The size of deletions in *ts* 6389 and *ts* 8741 was calibrated using vaccinia DNA digested with Ava I. This was possible because the fragments containing the deletion were 2×10^6 daltons and clearly separated away from other restriction fragments (Figure 4). Using as markers for calibration λ plac 5 digested with Eco RI and Hind III, the deletion in *ts* 6389 was estimated to be 0.14×10^6 daltons or 210 bp, while that in *ts* 8741 was <100 bp. The positions of the deletions within the vaccinia genome could be localized because there exists a fortuitous identity of the Hind III sites in IHD-W and the closely related sero-

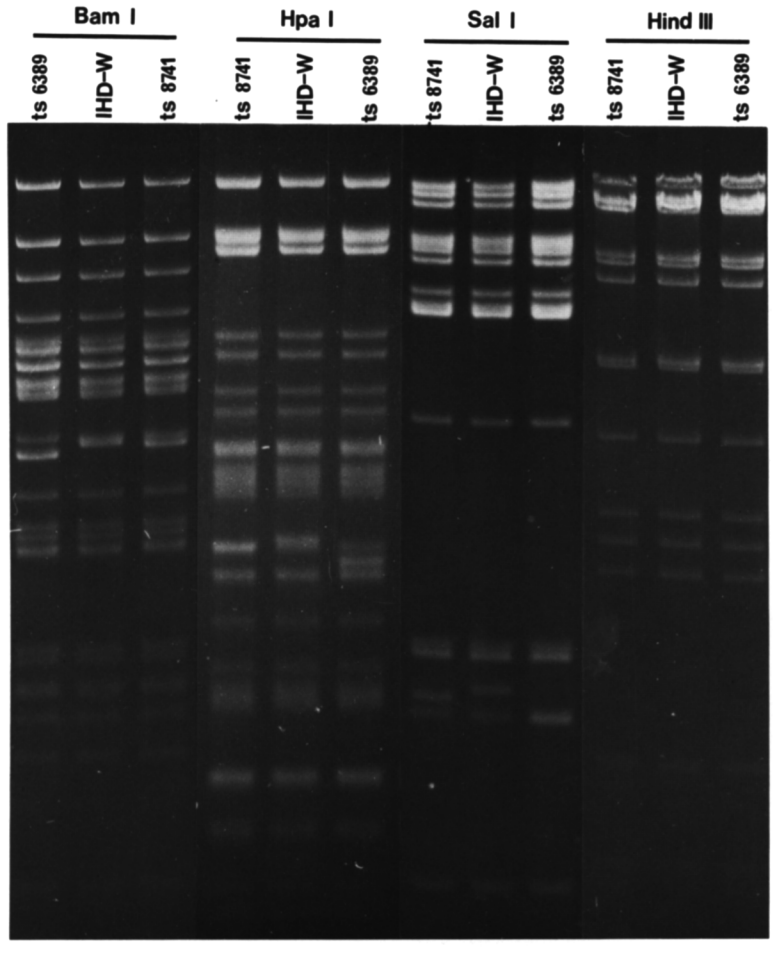

Figure 3. Bam I, Hpa I, Sal I and Hind III Digestion of Deletion Mutants *ts* 6389 and *ts* 8741

IHD-W, *ts* 6389 and *ts* 8741 were analyzed in 0.7% agarose gels as in Figure 2 after digestion with the various enzymes.

types of vaccinia examined by Garon et al. (1978) and McCarron et al. (1978). The homology established was especially useful inasmuch as digestion with Hind III enables one to distinguish the left and right ends of the genome, which contains inverted terminal repetitions of 7–8 × 10^6 daltons (Garon et al., 1978; Wittek et al., 1978a). When the three largest Hind III fragments were separated by means of very diluted (0.2%) agarose gels, in which migration of fragments <2 × 10^7 daltons varies linearly with the log of the molecular weight (Fangman, 1978), it appeared that both fragments B and C, derived from the two terminal repetitions, possessed marginally increased mobility in *ts* 6389 when compared with those of wild-type virus (Figure 5A). This was confirmed by extracting the Hind III fragments B and C and then digesting them with Eco RI (Figure 5B). The fact that fragments B and C of IHD-W share a common subset of three Eco RI fragments, adding up to a total molecular weight of 8.3 × 10^6 daltons, supports the presumption that they encode the two repetitive termini. It is quite clear that both the B and C fragments in *ts* 6389 created by Hind III lack the identical Eco RI segment, and therefore these deletions could not have arisen by a single alteration but must be, like the terminal sequences, of a diploid nature. *ts* 6389 genome is therefore shortened by a total of 420 bp, one half being deleted from each terminus.

The Deletions Are Not Related to the Temperature-Sensitive Phenotype

To evaluate the functional consequence of these deletions on virus growth, we attempted to ascertain whether they were associated exclusively with *ts* mutants defective in DNA replication. For this purpose, spontaneous ts^+ revertants of *ts* 6389 and 8741 were selected, isolated and propagated, and their DNA was subjected to Eco RI analysis. The data, shown in Figure 6, revealed that in all cases the same abbreviations of the genome as those identified in the parental, conditional lethal mutants were maintained. So far we have not observed any isolates in which the deleted sequences were restored. This implies either that the loss of a genome segment near each terminus was unrelated to the mutant phenotype, or that once created, the deletions could be suppressed by modifications in other parts of the genome. Evidence supporting the first alternative as the correct explanation

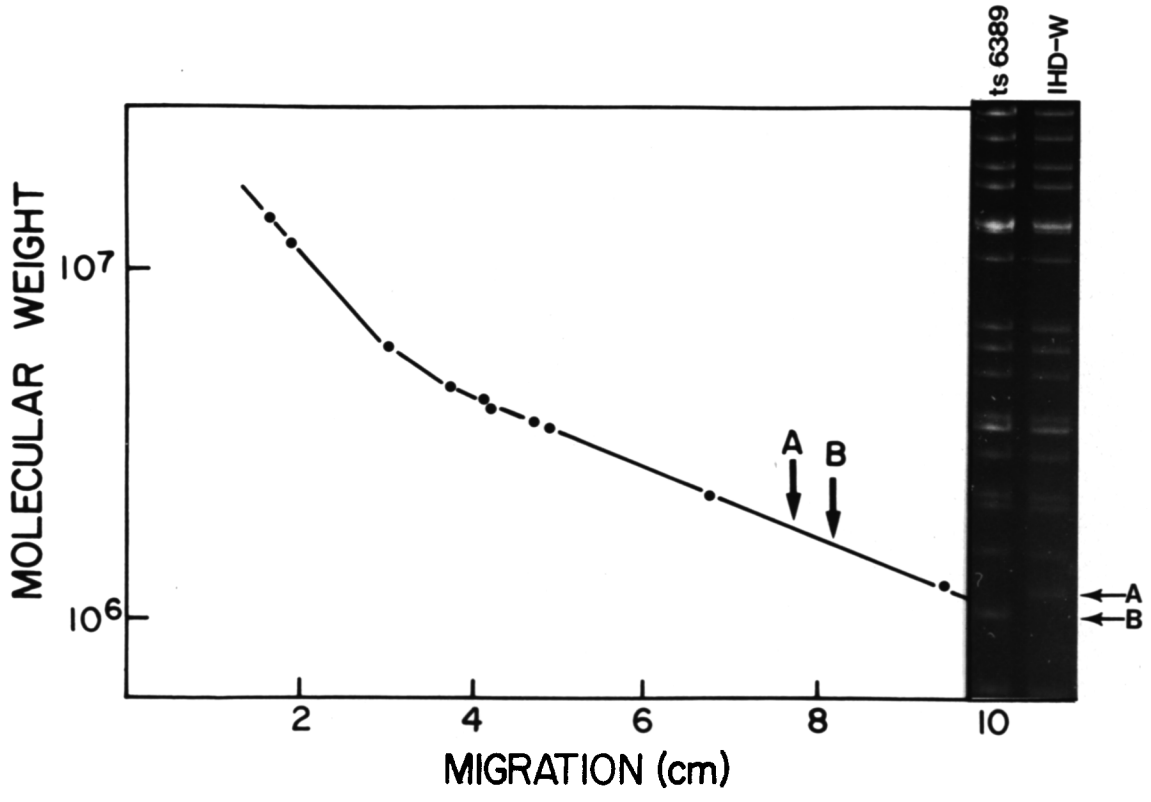

Figure 4. Calibration of Deletion Size in *ts* 6389

IHD-W and *ts* 6389 were analyzed in 0.7% agarose gels as in Figure 2 after digestion with Ava I. Molecular weight markers were the Eco RI and Hind III digests of λ plac 5 (Robinson and Landy, 1977). Arrows (A) and (B) refer to the positions of the wild-type and deletion-carrying fragments in IHD-W and *ts* 6389, respectively.

is based, first, on the fact that several among our late or DNA^+ mutants contain shortened segments in the identical Eco RI fragments (for example, in *ts* 9383), albeit the number of base pairs missing varies between an unknown minimum to a maximum of 250 bp for each terminus. Second, in the course of routine analysis of spontaneous revertants arising from various categories of *ts* mutants which possessed a pattern of fragments like that of IHD-W, it was observed that about one in five among these exhibited genome shortening of about the same size and at precisely the same position as the deletions evident in DNA^- mutants. From these facts, it can be concluded that loss of sequences near the termini of vaccinia DNA was unrelated to the mutagenesis procedures used for creating the *ts* mutants, but was rather the consequence of spontaneous and frequently occurring events during vaccinia replication. Nor were any phenotypic abnormalities evident among the ts^+ revertants acquiring the deletions when they were compared with functions expressed by wild-type IHD-W, indicating that the sequences deleted probably do not encode any function essential for the economy of vaccinia virus. It must be emphasized, and it may be of the utmost significance, that whenever deletions were detected, whether in the *ts* mutants or in revertants derived therefrom, they were never asymmetrical. Thus whatever the molecular events involved in causing the elimination of sequences, the deletions existed invariably as mirror-image alterations at both inverted termini.

Discussion

Restriction Endonuclease Analysis of *ts* Mutants and Mirror-Image Deletions

The molecular basis for *ts* mutations associated with various DNA animal viruses described previously (Fenner, 1969; Di Mayorca and Callender, 1970; Ghendon, 1972; Schaffer, 1975; Eckhart, 1977; Ginsberg and Young, 1976) remains obscure. To date, one isolate was found to possess an additional Eco RI cleavage site in the 5.8×10^6 dalton fragment D, and revertant analysis has implicated this alteration as the locus for the temperature-sensitive mutation (G. McFadden and S. Dales, manuscript in preparation).

During the course of these analyses, we unexpectedly observed that about one fifth of all the *ts* isolates possess small deletions ranging in size up to 250 bp.

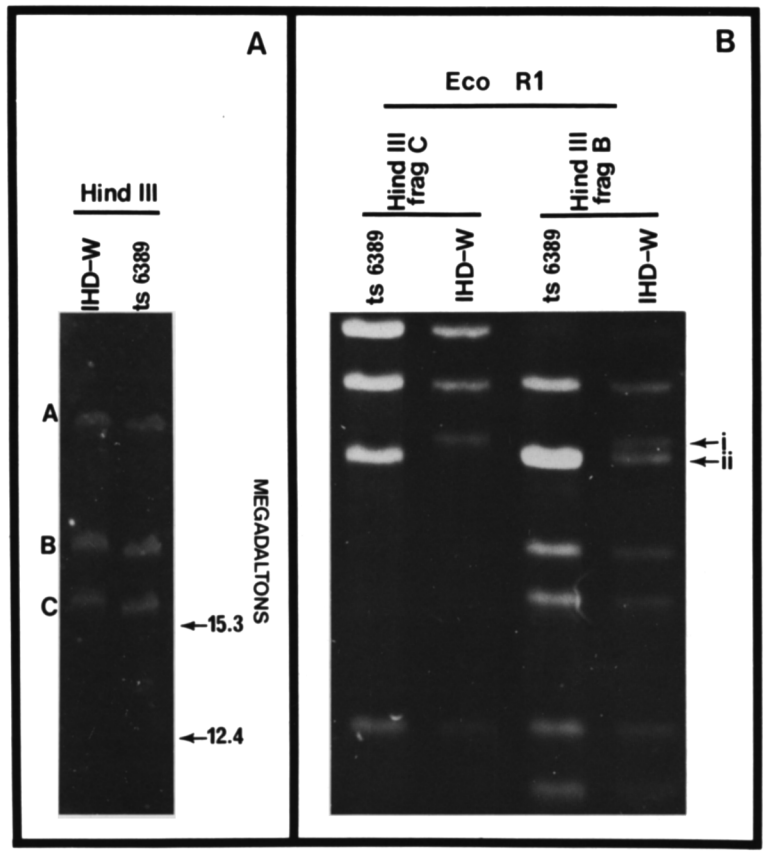

Figure 5. Analysis of Hind III Fragments A, B and C of IHD-W and *ts* 6389

(A) IHD-W and *ts* 6389 were digested with Hind III and analyzed by electrophoresis in 0.2% agarose. Arrows refer to the positions of the largest fragments of Eco RI- and Hind III-digested λ plac 5.

(B) Hind III fragments B and C were extracted from preparative 0.2% agarose gels, and the DNA was extracted and redigested with Eco RI and analyzed in 0.7% agarose gels as in Figure 2. Arrows (i) and (ii) refer to the positions of the wild-type and deletion-carrying fragments of intact IHD-W and *ts* 6389 after Eco RI digestion.

When mapped with restriction endonucleases, these deletions were always detected at the same location within inverted terminal repeats of the genome. In the IHD-W vaccinia, these repeated sequences are each 8.3×10^6 daltons in size, which compares with the size of such terminal fragments identified in other vaccinia strains by Garon et al. (1978) and Wittek et al. (1978a). It has been observed that some size heterogeneity exists in the terminally redundant sequences of uncloned vaccinia virus (Wittek et al., 1978b), suggesting that sequences in this segment of the genome are not highly conserved. This notion is supported by the finding that spontaneous mutants of cowpox called "white pock" variants exhibit a great deal of modulation within the terminal sequences (Archard, Mackett and Dumbell, 1978). It is worth noting that the size of the largest vaccinia deletions, ~250 nucleotide pairs per terminus, is approximately the same as the terminal "a" sequence of herpes simplex DNA (Roizman, 1979). This "a" sequence may occur in as many as 3 or 4 copies per terminus, but can vary in abundance upon cloning by plaque purification, whereby either an increase or decrease in the number of such copies results. The major difference with the type of deletion-insertion/isomerization observed with herpes and the modulation in the vaccinia genome reported here is that heretofore we have not detected any instance of reintroduction of the deleted sequences. We cannot, however, exclude the possibility that such reinsertions do occur very infrequently.

The unexpected finding in this study was the occurrence of small, spontaneous deletions, invariably located at a unique position but presumably of opposite orientation, in the terminal fragments of the IHD-W genome. We never observed a single instance where sequences were deleted at one terminus without showing the existence of a mirror-image deletion in the other terminus. Spontaneous generation of these unique deletions must be independent of the mutations associated with *ts* loci because first, deletions occur randomly among the various classes of our collection of *ts* mutants; second, all spontaneous ts^+ revertant clones isolated from deletion-carrying mutants retained such deletions; and third, some spontaneous ts^+ revertant clones arising from *ts* mutants lacking terminal deletions now possess them. In this category, isolates with deletions occur at about the same frequency as that observed with the *ts* mutants in general. The existence of these viable ts^+ revertants indicates that the deleted sequences do not code for obligatory functions. We conclude that the deletions are unrelated to the *ts* phenotype and are spontaneously generated by a single molecular event during poxvirus replication. A circular interme-

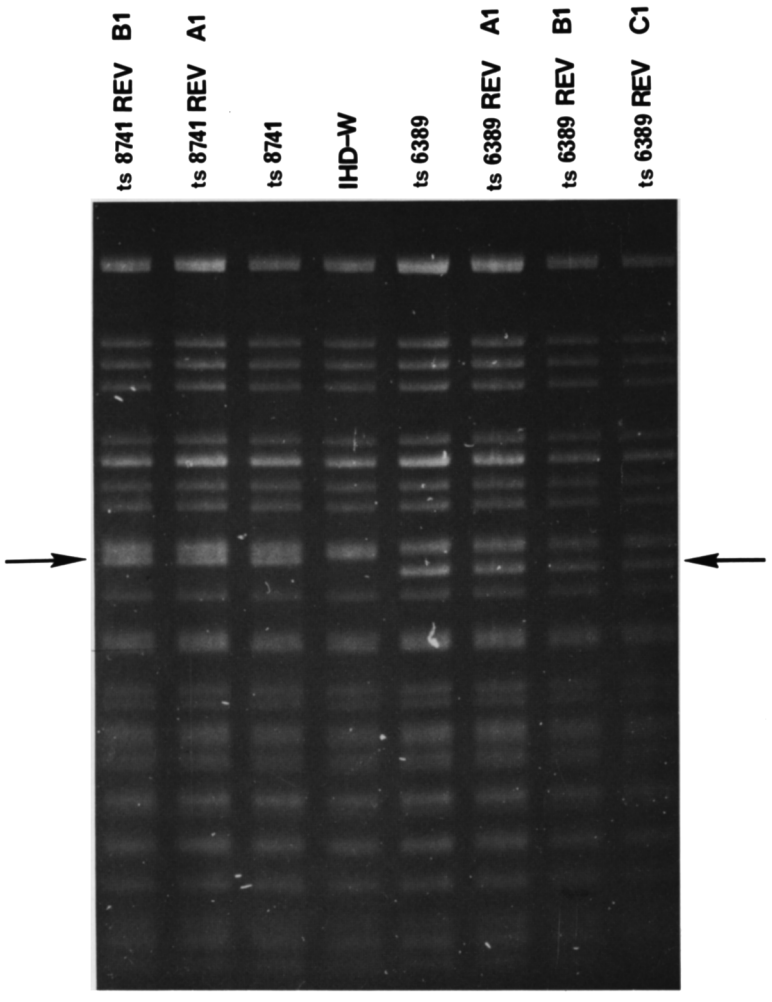

Figure 6. Analysis of Spontaneous ts$^+$ Revertants of ts 6389 and ts 8741

DNA from two spontaneous revertants of *ts* 8741 and three of *ts* 6389 (see Experimental Procedures) was extracted, digested with Eco RI and analyzed by electrophoresis in 0.7% agarose as in Figure 2. Arrows refer to the positions of the deletion-carrying fragments of *ts* 8741 and *ts* 6389.

diate is the only plausible structure in which the existence of juxtaposed ends of a linear molecule could give rise to mirror-image excisions.

Significance of Terminal Mirror-Image Deletions

Characterization of symmetrical excisions which appear spontaneously at high frequency in the vaccinia genome must be explained in terms of the mechanism of DNA synthesis. More specifically, the problem has not been resolved of how to replicate the terminal sequences of a linear duplex molecule by DNA polymerases able to synthesize only in the 5′ → 3′ direction from preexisting "primers" endowed with free 3′-hydroxyl groups. Although molecules which contain terminal repetitions, as in bacteriophages λ and T4, can circumvent this difficulty by their intrinsic capacity to form either circles or concatemers (Watson, 1972; Dressler, 1975), there are other DNA genomes which do not possess this type of terminal structure, as discussed in theoretical terms by Cavalier-Smith (1974), Bateman (1975) and Heumann (1976). Thus adenovirus possesses repetitious terminal sequences of approximately 100 bp which are of inverted orientation, therefore precluding the generation of continuous covalent circles or concatemers (Winnacker, 1978). By comparison, good evidence exists that both circular and concatemeric intermediates are formed in the case of herpes virus DNA (Friedmann and Becker, 1977; Becker et al., 1978; Jacob, Moise and Roizman, 1979).

Although vaccinia codes for a DNA polymerase whose mode of synthesis in vitro is unidirectional in the manner of other DNA polymerases (P. Englund, personal communication), the termini of the DNA are initially cross-linked (Geshlin and Berns, 1974), probably existing as hairpins (B. Pogo, personal communication). To incorporate this information into their model of replication, Esteban, Flores and Holowczak (1977) proposed that one of the cross-linked termini acted as a focus for the origin of a replication bubble which subsequently expanded to the other terminus. This model attempts to circumvent the problem of replicating terminal nucleotides without invoking the need for circular or concatemeric intermediates.

The data presented here strongly imply that vaccinia DNA may exist, at least transiently, in a circular con-

formation. While there is some indirect evidence for circular monomers of vaccinia DNA in infected cells (Holowczak and Diamond, 1976; Archard, 1979), the actual configuration of such intermediates is undetermined. Like poxviruses, adenovirus also contains inverted terminal repetitions, but circular intermediates of adeno DNA have been observed only in conjunction with a protein linker joining the ends (Robinson, Younghusband and Bellett, 1973; Robinson and Bellett, 1974; Girard et al., 1977), so that the role of the repeated terminal sequences in circularization is only a conjecture (Winnacker, 1978). However, the association between terminal repetitions and the generation of deletions during circularization reactions is very striking in other virus systems (Shank et al., 1978a, 1978b; Yoshimuna and Weinberg, 1979), lending credence to the idea that circularization may be an important facet of poxvirus replication. Whether such circularization is mediated by the inverted terminal sequences or by a protein linker, as in the case of adenoviruses, is unknown.

Experimental Procedures

Cells and Virus

Mouse L cells, vaccinia IHD-W and the *ts* mutants derived therefrom were grown as described by Dales et al. (1978). Spontaneous revertants of the *ts* mutants were generated by directly plating viral suspensions on monolayers at 39.5°C and allowing plaques to develop for 2–4 days in minimal Eagle's medium with 10% fetal calf serum plus 0.5% methylcellulose A4M (Dow Chemical). Isolates were twice cloned and retitrated to screen for any temperature sensitivity; all revertants described here were indistinguishable from wild-type virus in their 32.5°/39.5°C plaquing efficiency.

DNA Extraction

L cells were infected with a multiplicity of 5–10 pfu per cell of the various *ts* mutants, and labeled for 24 hr either with 0.1 μCi/ml methyl-^{3}H-thymidine (New England Nuclear) for preparative extractions, or with 0.1 μCi/ml methyl-^{14}C-thymidine plus 1 μCi/ml ^{3}H-l-amino acid mixture (New England Nuclear) for analytical analysis. The virus was then purified from cytoplasmic extracts by centrifugation in potassium tartrate gradients according to published procedures (Stern and Dales, 1974). Viral DNA was extracted from the purified vaccinia virions by a modification of the procedure of Parkhurst and Heidelberger (1976). Virus at a concentration of 10^{11} elementary bodies per ml in 10 mM Tris–HCl (pH 8) was layered onto 39 ml 5–20% sucrose gradients containing 2% β-mercaptoethanol, 1% sodium dodecylsulphate, 100 mM NaCl, 1 mM EDTA and 10 mM Tris–HCl (pH 8), and subsequently overlaid with an identical lysis buffer but without sucrose. After incubating for 0.5 hr at room temperature, the samples were centrifuged for 4.5 hr at 26,000 rpm at 20°C in an SW27 rotor (Beckman), and fractions were collected from the bottom of the tube. For analytical determinations, fractions were precipitated with trichloroacetic acid in the presence of 50 μg/ml calf thymus DNA carrier onto glass fiber filters, washed with 5% TCA and 95% ethanol, and dried, and the radioactivity was determined in toluene-Omnifluor (NEN). For preparative purposes, aliquots were analyzed for radioactivity, and the fractions containing viral DNA were pooled, diluted with 10 mM Tris–HCl (pH 8), 1 mM EDTA, precipitated 2–3 times with 2 vol isopropanol plus 1/10 vol 3 M sodium acetate (pH 5.5), and finally resuspended at 100 μg/ml in 10 mM Tris–HCl (pH 8), 0.1 mM EDTA.

Restriction Enzyme Digestions

Digestions were carried out in 20 μl reactions at 37°C under the following conditions: Eco RI (Miles)–100 mM Tris–HCl (pH 7.5), 50 mM NaCl, 10 mM $MgCl_2$; Sal I (Miles)–8 mM Tris–HCl (pH 7.5), 150 mM NaCl, 6 mM $MgCl_2$, 0.2 mM EDTA, 100 μg/ml bovine serum albumin; Hind III (Boehringer-Mannheim)–10 mM Tris–HCl (pH 7.6), 50 mM NaCl, 10 mM $MgCl_2$, 14 mM dithiothreitol; Ava I (Bethesda Research)–20 mM Tris–HCl (pH 7.4), 30 mM NaCl, 10 mM $MgCl_2$; Hpa I (Bethesda Research)–20 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 6 mM KCl, 1 mM dithiothreitol. Reactions were stopped by adding 10 μl 50% sucrose, 0.125% bromophenol blue, 50 mM EDTA, 10 mM Tris–HCl (pH 8).

Agarose Gel Electrophoresis

Electrophoresis of viral DNA (0.5–2 μg per well) was carried out in a horizontal gel apparatus at 50–75 V for 12–20 hr at room temperature. Agarose (Seakem ME) was dissolved at 0.2–0.7% in electrophoresis buffer, 40 mM Tris–acetate (pH 8), 20 mM sodium acetate, 1 mM EDTA, and autoclaved before pouring. After electrophoresis, the gel was stained with 1 μg/ml ethidium bromide, and the DNA bands were visualized with a Black-Ray ultraviolet transilluminator (Ultra Violet Products) and photographed through a Kodak No. 23 yellow filter on Kodak high-contrast copy film. λ plac 5 DNA (a gift from G. Mackie) was digested with either Eco RI or Hind III and used as a molecular length marker for all electrophoresis runs (Robinson and Landy, 1977). For purification of fragments from gels for further enzyme digestion, the DNA bands were excised and purified from contaminating agarose by the method of Vogelstein and Gillespie (1979).

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