The Genome Structure of Cowpox Virus White Pock Variants

By L. C. ARCHARD,* M. MACKETT,† D. E. BARNES‡ AND K. R. DUMBELL§

Division of Virology, Department of Medical Microbiology, St Mary's Hospital Medical School, Praed Street, London W2 1PG, U.K.

(Accepted 14 February 1984)

SUMMARY

A previous report described restriction endonuclease analysis of white pock variants of red cowpox virus and their characterization as deletion mutants lacking certain sequences including the repetition from one specific terminus of the wild-type genome. Further analysis has confirmed the terminal deletion but demonstrated that this is compensated at the site of deletion by the presence of an inverted duplication of a variable amount of sequence from the opposite terminus, with the effect of restoring a terminal repetition and the covalent, terminal crosslink. Nine of 11 white pock variants showed a similar deletion of about 21 Mdal mapping contiguously from the right-hand terminus and extending into a 2-4 Mdal restriction fragment. Two white variants showed larger deletions of about 24 and 27 Mdal respectively. These deletions were compensated by a copy of sequences from the opposite terminus which ranged in size from 3 to 27 Mdal. No terminal deletions smaller than 21 Mdal were observed in cowpox white variants or in clones retaining the red phenotype. In contrast with other orthopoxviruses, no deletions involving the left-hand terminus were found. Some independent white isolates had similar sizes of sequence copied from the opposite terminus, but some sibling clones from a single, pock-purified white isolate with the same size of deletion had different sizes of duplicated sequence. Other siblings isolated from an independent, three times pock-purified white clone, itself derived from a single parental red pock, differed from each other in the size of both the deletion and the duplicated sequence. These observations suggest preference for deletion in a particular region, conjunction of the genome termini during DNA replication and a requirement for the preservation of symmetrical termini in orthopoxvirus genome function.

INTRODUCTION

Restriction endonuclease cleavage site maps permit comparison of DNA genomes from normal and mutant viruses and thus the possibility of correlation of gene functions with DNA loci. Wild-type cowpox viruses normally produce haemorrhagic, ulcerated lesions (pocks) on the chorioallantois of fertile hens' eggs, but white pock variants arise with a frequency of about 1% (Downie & Haddock, 1952; van Tongeren, 1952): these have some characteristics suggestive of deletion mutants (Rondle & Dumbell, 1962; Baxby & Rondle, 1968).

White pock variants of pock-purified red cowpox strain Brighton were shown by restriction endonuclease mapping to be deletion mutants lacking similar sequences from one specific terminus of the parental genome; these sequences included restriction fragments containing the terminal repetition originally present (Archard & Mackett, 1979). Further investigations were prompted by the apparent lack of requirement for the terminal repetition in replication of...
mutant DNA, the inability to detect a covalent crosslink at the site of deletion and by the apparent similarity of genome structure in some mutants isolated independently. Restriction fragments mapping at the non-deleted terminus are present in over 1-molar amounts in DNA from these mutants and such reiterated terminal sequences are present as a free end (see below). It was hypothesized that these sequences are copied to the opposite, deleted terminus with the effect of preserving an inverted terminal repetition as was proposed for certain monkeypox mutants (Dumbell & Archard, 1980) or, that such sequences are acquired by recombination with similar effect. White pock variants of rabbitpox were shown by Moyer et al. (1980) to have genomes rearranged in this way and the genome structure of analogous monkeypox white mutants has been confirmed by Esposito et al. (1981). Analysis of DNA from the initial Brighton cowpox white pock isolates showed that some restriction fragments, thought previously to represent partial digestion products, were present in submolar amounts. As these mutants undergo further variation in the course of propagation for stock, subclones were prepared from one of the white pock isolates described previously (Archard & Mackett, 1979). The results of analysis of two such subclones designated A or C and of nine other, independent white cowpox isolates are presented here and demonstrate that, as for rabbitpox and monkeypox mutants, a copy of sequences similar to those constituting the non-affected terminus is linked covalently to the site of deletion with the effect of restoring an inverted terminal repetition and terminal covalent crosslink and thus maintaining the symmetry of the virus genome. In contrast with rabbitpox mutants having analogous genome rearrangements (Moyer et al., 1980), the deletion in all cowpox white variants examined is from the same terminus and, in the majority of these mutants, extends into the same small restriction fragment which represents less than 2% of the wild-type virus genome.

METHODS

Methods were generally as described previously (Archard & Mackett, 1979) with the following variations.

Virus growth and purification. The isolation and propagation of white pock variants from pock-purified red cowpox strain Brighton were described previously. In the present study, further single pock isolates from these initial, white pock variants or from clones with the red phenotype were prepared by successive cycles of pock purification by passage at high dilution on the chorioallantois of 12-day, fertile hens’ eggs and filtration through 650 nm Millipore filters (Dumbell & Archard, 1980) followed by propagation in monolayer cultures of primary chick embryo fibroblasts (CEF). Virus was purified from supernatants after low-speed centrifugation of infected CEF disrupted by swelling at 4 °C in 5 mM-Tris–HCl pH 7-4 and minimal ultrasonic treatment (Megasonic, 80 kHz) as described previously (Joklik, 1962; Mackett & Archard, 1979).

Cleavage of DNA. DNA was isolated from purified virus, cleaved with restriction endonuclease SfiI, BgII, HindIII, XhoI, PstI or KpnI under standard conditions and fragments were separated by electrophoresis as described previously (Mackett & Archard, 1979).

Estimation of molarity of restriction fragments. Restriction fragments separated by electrophoresis in agarose slab gels and stained with ethidium bromide (0.5 µg per ml of electrophoresis buffer) were photographed by u.v. transillumination. The resulting negatives were scanned with an integrating densitometer (Quick Scan, Helena Laboratories, Beaumont, Tex., U.S.A.) and relative molarities of individual fragments were estimated by reference to fragment size.

Isolation of DNA fragments, in vitro labelling of DNA and DNA–DNA hybridization. Restriction fragments located by minimal ethidium bromide staining were recovered from gels by ‘freeze–squeeze’ (Thuring et al., 1975), labelled with 32P by nick translation essentially as described by Rigby et al. (1977) and hybridized to restriction fragments immobilized on nitrocellulose (Southern, 1975; Jeffreys & Flavell, 1977).

Identification of covalently crosslinked restriction fragments. Covalently crosslinked, presumed terminal, restriction fragments (Geshelin & Berns, 1974; Wittek et al., 1977) were detected by ethidium bromide staining following electrophoresis in parallel with normal digests (Archard & Mackett, 1979).

RESULTS

Comparison of the restriction products of DNA from parental red cowpox or white pock variants

DNA from Brighton red cowpox (Brcp) or from white pock variants (Bwcp) was cleaved with restriction endonuclease HindIII, XhoI, PstI or KpnI and the fragments were separated by agarose slab gel electrophoresis. Virus-specific fragments in Fig. 1 are identified by lettering in
Cowpox virus mutant genome structure

Fig. 1. *HindIII, Xhol, PstI* or *KpnI* restriction fragments of DNA from wild-type red cowpox strain Brighton (BR), a white pox variant described previously (OW; Archard & Mackett, 1979) or white variant subclones A (CIA) or C (CIC) separated by agarose gel electrophoresis. Restriction fragments of wild-type DNA absent from digests of DNA from white variants are indicated by arrows; duplicated or unique fragments from DNA of white variants are indicated by bars or asterisks respectively.
Fig. 2. Densitometric scans of ethidium bromide-stained *Pst*I restriction fragments of DNA from wild-type red cowpox strain Brighton (a), white variant clone A (b) or clone C (c) after separation by agarose gel electrophoresis. restriction fragments are lettered in order of decreasing molecular mass. The total amount of DNA in a band and thus the molarity in relation to fragment size is a function of the area under a peak.

Table 1. *Molecular mass (Mdal) of Brcp restriction fragments absent from digests of Bwcp DNA* 

<table>
<thead>
<tr>
<th>HindIII</th>
<th>XhoI</th>
<th>PstI†</th>
<th>KpnI</th>
</tr>
</thead>
<tbody>
<tr>
<td>H</td>
<td>6.6</td>
<td>E</td>
<td>11.8</td>
</tr>
<tr>
<td>L</td>
<td>5.1</td>
<td>O</td>
<td>2.5</td>
</tr>
<tr>
<td>B</td>
<td>17.0</td>
<td>L</td>
<td>3.3</td>
</tr>
<tr>
<td>J</td>
<td>7.4†</td>
<td>D</td>
<td>12.0</td>
</tr>
</tbody>
</table>

* Fragments are lettered in order of decreasing molecular mass (Mdal). See Fig. 4 for map locations.
† Two-molar bands co-migrating with the 2-molar Brcp terminal fragments *Pst*I K are present in *Pst*I digests of DNA from most Bwcp variants (see Results).
‡ *Pst*I J is absent from a minority of variants.
Fig. 3. Genome structure proposed for the cowpox white pock variants described here. (a) Cross-hatched regions represent inverted terminal repeats in the wild-type genome, shaded regions represent left-hand unique sequences, non-shaded regions represent right-hand unique sequences and arrows represent hypothetical restriction sites. A right-hand terminal deletion (b) is compensated by the presence of an inverted duplication of sequences mapping at the opposite terminus (c). Restriction endonuclease cleavage generates 2-molar, apparent left-hand terminal fragments and unique junction fragments containing sequences originating from different regions of the wild-type genome.

order of decreasing molecular weight, and physical maps showing the size and location of Brcp fragments resulting from cleavage with HindIII, XhoI, PstI or KpnI have been presented previously (Archard & Mackett, 1979). Comparison of restriction products of Brcp DNA with those of DNA from Bwcp isolates demonstrates in white variants the absence of DNA sequences mapping contiguously from the right-hand terminus of the wild-type genome (arrows, Fig. 1; Table 1; Fig. 4). Similar wild-type restriction fragments are absent from DNA of nine of 11 white isolates and, with other data (see below), indicate a terminal deletion of between 20 Mdal and 22 Mdal compared with wild-type DNA. DNA from two white variants lacked the additional contiguous restriction fragments PstI Q and PstI J, indicating a larger deletion.

Most restriction profiles of DNA from white pock variants contain fragments present in over 1-molar amounts, as detected by enhanced staining with ethidium bromide (bars, Fig. 1) and quantification by densitometry (Fig. 2). The fragments concerned represent the left-hand terminus of the wild-type genome and sequences mapping contiguously in from this in each case (Fig. 4).

The absence of sequences mapping at one terminus of the wild-type genome, with the reiteration of sequences representing the opposite terminus duplicated as a free end suggests that the genome structure of these white pock variants results from a terminal deletion which is compensated by the presence of sequences identical to those mapping at the non-deleted terminus (Fig. 3). The model proposes the restoration of an inverted terminal repetition either larger or smaller than that of the wild-type, with a terminal covalent crosslink and predicts the existence of unique hybrid junction fragments containing sequences derived from opposite ends of the wild-type genome. In contrast with the initial Bwcp isolates (Archard & Mackett, 1979) restriction digests of DNA from white subclones A or C show new discrete fragments (asterisks, Fig. 1). Some of these (e.g. clone A/HindIII G; KpnI D: clone C/HindIII B; PstI L) co-migrate
Table 2. Molecular mass (Mdal) of deleted sequence, duplicated sequence, unique junction fragment generated by restriction endonuclease cleavage and total genome of white pock variants

<table>
<thead>
<tr>
<th>White variant</th>
<th>Size of deleted sequence</th>
<th>Size of duplicated sequence</th>
<th>Size of unique junction fragment</th>
<th>Size of genome</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A 211</td>
<td>21</td>
<td>3</td>
<td>10 40 3 4</td>
<td>128</td>
</tr>
<tr>
<td>Clone C</td>
<td>21</td>
<td>7</td>
<td>14 40 3 4</td>
<td>132</td>
</tr>
<tr>
<td>3A 12</td>
<td>21</td>
<td>14</td>
<td>10 40 9 15</td>
<td>136</td>
</tr>
<tr>
<td>6A 222</td>
<td>24</td>
<td>14</td>
<td>6 40 12 15</td>
<td>136</td>
</tr>
<tr>
<td>6A 61</td>
<td>21</td>
<td>16</td>
<td>12 40 15 8</td>
<td>141</td>
</tr>
<tr>
<td>3A 13</td>
<td>27</td>
<td>18</td>
<td>7 40 6 15</td>
<td>137</td>
</tr>
<tr>
<td>6A 172</td>
<td>21</td>
<td>25</td>
<td>21 40 11 13</td>
<td>150</td>
</tr>
<tr>
<td>Clone A</td>
<td>21</td>
<td>26</td>
<td>26 40 11 13</td>
<td>151</td>
</tr>
<tr>
<td>3A 2</td>
<td>21</td>
<td>27</td>
<td>7 40 12 13</td>
<td>152</td>
</tr>
<tr>
<td>6A 623</td>
<td>21</td>
<td>27</td>
<td>6 40 12 13</td>
<td>152</td>
</tr>
</tbody>
</table>

* The terminal deletions extend into the 40 Mdal wild-type XhoI A fragment which consequently generates large junction fragments whose size is difficult to determine by agarose gel electrophoresis.

Table 3. Hybridization of \(^{32}P\)-labelled Bwcp unique HindIII fragments to immobilized Bwcp or Brcp restriction fragments

<table>
<thead>
<tr>
<th>Bwcp (^{32}P)-probe</th>
<th>Homologous Bwcp clone HindIII</th>
<th>Brcp HindIII</th>
<th>XhoI</th>
<th>PstI</th>
<th>KpnI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone A/HindIII fragment G</td>
<td>G</td>
<td>B</td>
<td>A</td>
<td>C</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>N*</td>
<td>P</td>
<td>F</td>
<td>J</td>
<td>D</td>
</tr>
<tr>
<td>Clone C/HindIII fragment B</td>
<td>B</td>
<td>D</td>
<td>A</td>
<td>F</td>
<td>A/B</td>
</tr>
<tr>
<td></td>
<td>D†</td>
<td>H†</td>
<td>D</td>
<td>J</td>
<td>C</td>
</tr>
<tr>
<td></td>
<td>H†</td>
<td>E†</td>
<td>K†</td>
<td>D</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q</td>
<td>O</td>
<td>H†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Bwcp clone A/HindIII N is the fragment equivalent to Brcp/HindIII P (Fig. 4).
† Brcp fragments representing the right-hand terminus (deleted in Bwcp isolates) demonstrating that the probe also contains sequences from the wild-type terminal repetition (Fig. 4).

with submolar bands thought previously to be partial digestion products of Bwcp DNA but now shown to result from cleavage of minority species of genome selectable by subcloning. Similarly, unique restriction fragments are present in digests of DNA from further cowpox white variants isolated after repeated cycles of pock purification. The sizes of unique fragments in different restriction enzyme digests of DNA from particular clones (Table 2) are not compatible with simple deletion of sequences from one terminus of the wild-type genome.

Origin of unique restriction fragments in digests of DNA from white pock variants

Various unique restriction fragments were isolated from DNA of Bwcp clone A or C and \(^{32}P\)-labelled by nick translation. These probes were tested for sequence homology with wild-type DNA by hybridization to HindIII, XhoI, PstI or KpnI fragments of Brcp DNA separated by electrophoresis and transferred to nitrocellulose filters. The specificity of individual probes was determined by hybridization to the homologous transfer. Hybridizations obtained with the probe clone A/HindIII fragment G or clone C/HindIII fragment B are summarized in Table 3 and demonstrate that such unique restriction fragments from white variant DNA contain sequences that map at disparate loci in the wild-type genome. For example, Bwcp clone A/HindIII G hybridizes specifically with both Brcp/HindIII B (or the colinear fragment
Cowpox virus mutant genome structure

Fig. 4. Physical map locations of HindIII, XhoI, PstI or KpnI restriction fragments of DNA from wild-type red cowpox strain Brighton (Brcp) or representative white pock variants: clone A (CIA), clone C (CIC) and 6A 211. Fragments are lettered in order of decreasing molecular mass. Boxes indicate the size of deletion from the right-hand terminus of the wild-type genome. Underlinings indicate the size of sequence duplicated from the left-hand terminus generating 2-molar and unique junction fragments after restriction endonuclease cleavage. Cleavage sites for endonuclease BglI or SstII in Brcp DNA are also shown.

Brcp/XhoI A) mapping near the right-hand terminus and with Brcp/HindIII P (or the colinear fragment Brcp/XhoI F) mapping near the left-hand terminus as drawn in Fig. 4. Analogous results were obtained by probing with the Bwcp clone A unique KpnI fragment D (data not shown) which hybridizes also with clone A/HindIII G.

Unique restriction fragments from DNA of Bwcp clone C have sequence homology with Brcp fragments mapping at the same, near right-hand terminal location but also with near left-hand terminal wild-type fragments mapping at a locus different from that seen with Bwcp clone A probes. For example, Bwcp clone C/HindIII B again hybridizes specifically with Brcp/HindIII B (or Brcp/XhoI A) but also with the left-hand terminal fragment Brcp/HindIII D or the colinear fragment Brcp/XhoI D (Table 3; Fig. 4). This probe hybridizes also to the Brcp fragments XhoI E and KpnI H representing the opposite terminus (deleted in Bwcp isolates), indicating that it includes sequences from the wild-type terminal repetition (Archard & Mackett, 1979).

These data confirm that unique restriction fragments from DNA of white pock variants contain junctions between the point of deletion of Brcp right-hand terminal sequences and a variable amount of wild-type sequences representing a duplication of the opposite left-hand terminus.

Construction and analysis of physical maps

Physical maps of the cleavage sites for endonuclease HindIII, XhoI, PstI or KpnI in DNA of wild-type Brcp were presented previously (Archard & Mackett, 1979) and are reproduced here (Fig. 4) with modifications and the addition of sites for BglI or SstII resulting from further
experiments (data not shown). The map locations of Brcp restriction fragments absent from Bwcp DNA and the presence of sequences homologous with Brcp/PstI fragment O in Bwcp unique fragments indicate a similar, right-hand terminal deletion in nine of 11 white variants which extends to within the Brcp/PstI O fragment. Summation of the molecular weights of the contiguous Brcp/PstI fragments K, L and G gives the size of the deletion as about 20 Mdal plus some portion of the 2-4 Mdal Brcp/PstI O fragment. This value is compatible with the changes seen in other restriction endonuclease digests of DNA from these white pock variants. Two further white pock isolates had larger deletions extending into the PstI J fragment.

The map locations and sizes of Brcp restriction fragments duplicated in Bwcp DNA together with the homology between Brcp sequences mapping contiguously with these and the Bwcp unique fragments indicate that an inverted copy of the unaffected terminus is linked physically to the site of deletion thus generating hybrid junction fragments. Summation of the molecular weights of, for example, Brcp/HindIII fragments D and C gives the size of the duplication in Bwcp clone A as 25 Mdal plus some portion of the 1.4 Mdal Brcp/HindIII P fragment. This value is compatible with the sizes of the duplicated and unique fragments seen in other digests of DNA from Bwcp clone A and indicates a genome molecular weight approximately 4 Mdal greater than that of the wild-type. Similar considerations show that the size of the sequence duplicated from the left-hand terminus, and thus the size of the inverted terminal repetition in the white variants described, ranged from 3 to 27 Mdal plus some portion of the 1.4 Mdal Brcp/HindIII P fragment. This value is compatible with the sizes of the duplicated and unique fragments seen in other digests of DNA from Bwcp clone A and indicates a genome molecular weight approximately 4 Mdal greater than that of the wild-type. Similar considerations show that the size of the sequence duplicated from the left-hand terminus, and thus the size of the inverted terminal repetition in the white variants described, ranged from 3 to 27 Mdal plus some portion of the 1.4 Mdal Brcp/HindIII P fragment. This value is compatible with the sizes of the duplicated and unique fragments seen in other digests of DNA from Bwcp clone A and indicates a genome molecular weight approximately 4 Mdal greater than that of the wild-type.

The wild-type inverted terminal repetition contains a site for endonuclease PstI which thus generates an identical fragment (Brcp/PstI K; 6.3 Mdal; 2-molar) from each terminus (Archard & Mackett, 1979). The duplicated left terminal sequences in most white pock isolates extend past this site (Fig. 4) and so all PstI digests except of DNA from mutant 6A 211 contain a 2-molar fragment co-migrating with Brcp/PstI K, despite this sequence being deleted from the right-hand terminus of the white variants (Table 1).

Occurrence of other deletions

The high frequency of generation of white pock variants having a right-hand terminal deletion extending to within the PstI fragment O (about 1% of progeny from a wild-type clone) suggests a predilection for deletion at this locus. To test the possibility of deletions in this region being selected by their effect on a specific gene function controlling the ability to suppress leukocyte infiltration of the egg membrane lesion and so allowing the generation of a white pock, we examined the restriction profiles of DNA from 21 pock-purified clones with a red phenotype. All appeared to have the parental genotype (data not shown). Thus, no left-hand terminal deletions nor any right-hand terminal deletions shorter than 21 Mdal have been seen in DNA from cowpox virus clones having either the red parental or white variant phenotype.

Occurrence of terminal crosslinks

Digests of DNA from Bwcp isolates in which the sequence copied from the left-hand terminus extends past the first restriction site contain the wild-type, left-hand terminal restriction fragment in 2-molar amounts (Table 2; Fig. 1; Fig. 4). This indicates that reiterated sequences include a free left terminus and raises the question of a crosslink at this duplicated terminus. After formamide denaturation, the 2-molar terminal fragment in such digests renatures readily to an extent which suggests that all components of these bands are covalently crosslinked, although quantification by densitometry is equivocal as the efficiency of renaturation is reduced by nicks within the terminal restriction fragments. The situation may be resolved by considera-
Fig. 5. Native (a) or rapidly renaturing (b) HindIII restriction fragments of DNA from wild-type red cowpox strain Brighton (Brcp) or white variant clone C (CIC) separated by agarose gel electrophoresis.

tion of digests in which the sequences duplicated do not include a restriction site, e.g. Bwcp clone C/HindIII (Table 2; Fig. 4). In this case, the unique fragment is also the right-hand terminus (Table 3; Fig. 4) and shows rapid renaturation (Fig. 5). Thus, the effect of reiteration of wild-type, left terminal sequences at the deleted right-hand terminus of Bwcp DNA is to restore both an inverted terminal repetition and a covalent, terminal crosslink.

DISCUSSION

Restriction endonuclease cleavage maps for a variety of wild-type orthopoxviruses were published previously (Mackett & Archard, 1979) and demonstrate conserved or variable features in genome structure. In general, the central region of the genome is highly conserved between orthopoxviruses but is flanked by near-terminal sequences that are species-specific and are frequently the sites of variation in different strains of the same orthopoxvirus species. The wild-type genomes terminate in covalent crosslinks and have inverted, terminal repetitions which, although species-specific, generally share a subset of common sequences. The terminal repetitions contain multiple direct repeats interrupted by unique sequences (Wittek & Moss, 1980; Pickup et al., 1982).

Comparison of mutant with wild-type genome structures is an approach to mapping DNA functions and to determining the mechanism by which certain mutants arise. Orthopoxviruses that normally produce haemorrhagic lesions on chick chorioallantois offer a convenient selection for mutants in the form of white pock variants which arise with a frequency of 1%
(cowpox: Downie & Haddock, 1952) or less (monkeypox: Von Magnus et al., 1959; rabbitpox: Gemmell & Fenner, 1960). White pock variants of cowpox (Archard & Mackett, 1979), monkeypox (Dumbell & Archard, 1980) or rabbitpox (Lake & Cooper, 1980; Moyer & Rothe, 1980) have been investigated by restriction endonuclease analysis and some mutants in each case have been shown to be generated by deletion of terminal sequences relative to the wild-type genome. In other mutants, variations in terminal, repetitive sequences occur symmetrically at each end of the genome (McFadden & Dales, 1979), a situation which may extend beyond the original repetition to include previously unique sequences and suggesting that near terminal configurations are determined in the same process or that sequences at one end are copied to the other (Dumbell & Archard, 1980).

Archard & Mackett (1979) observed that deletions in white pock variants of cowpox included the wild-type repetition and the covalent crosslink from one specific terminus and concluded erroneously that these were not required for DNA replication. The present report establishes that, in fact, the genomes of these cowpox mutants retain an inverted, terminal repetition with terminal, covalent crosslink and that these arise by conjunction of the site of deletion with an inverted copy of sequences present at the opposite, unaffected terminus. This is demonstrated by the existence of unique restriction fragments from white pock variant DNA which contain a junction between sequences present in the wild-type genome at disparate loci and by duplication of the opposite terminus and sequences contiguous with it. These cowpox mutant genome structures are analogous to those reported previously for white pock mutants of rabbitpox (Moyer et al., 1980), certain monkeypox white mutants (Dumbell & Archard, 1980; Esposito et al., 1981) and some vaccinia virus mutants (M. Mackett & K. R. Dumbell, unpublished). It seems likely that mutants with genome rearrangements of this nature can be derived from all orthopoxvirus species.

For rabbitpox at least, the white pock phenotype can be generated by deletion at either terminus; in general, deletions involving the rabbitpox left terminus (Wittek et al., 1977) result in host range changes and those involving the right terminus do not (Lake & Cooper, 1980; Moyer & Rothe, 1980). In both cowpox and monkeypox, all such compensated deletions seen in white variants involve the right-hand terminus alone and representative cowpox white variants with the genome structure described here show no growth changes in a variety of cell types when compared to the wild-type. However, monkeypox white variants in which extensive sequence rearrangements occur symmetrically at both termini of the genome acquire the ability to grow in pig embryo kidney cells (Dumbell & Archard, 1980), suggesting that host range is controlled by sequences mapping near the left terminus of orthopoxvirus genomes.

The majority of right-hand terminal deletions characteristic of cowpox white variants extend to within the wild-type PstI fragment O, suggesting a preference for deletion at this locus. No left-hand deletions nor any right-hand deletions shorter than 21 Mdal were seen in DNA from cowpox clones having either wild-type or white variant phenotype.

In contrast, the size of the inverted copy of left terminal sequences found in conjunction with the deleted right terminus varied from 3 Mdal to 27 Mdal in this series of white pox isolates compared to the wild-type terminal repetition of about 6 Mdal. In cases capable of unequivocal determination, the hairpin structure crosslinking the DNA duplex at the non-affected terminus was also copied to the deleted terminus with the effect of restoring the overall symmetry of the genome. The apparent importance of symmetrical termini in orthopoxvirus genome functions is emphasized by the observation that, in a series of intertypic recombinants generated in vivo by mixed infection, all mapped as double crossovers having identical terminal sequences derived from one parent only (M. Mackett & K. R. Dumbell, unpublished results).

The provenance of cowpox white variants remains contentious. Single white pocks picked from chick chorioallantois after infection with pock-purified red cowpox stock at high dilution and passed once in primary CEF frequently generate a genotypically mixed population of virus as analysed by restriction endonuclease cleavage (Williamson & Mackett, 1982). It seems likely that a variable amount of left terminal sequence present at the deleted right terminus generates heterogeneous junction fragments not readily detectable as discrete bands by gel electrophoresis. Repeated single pock picking combined with filtration of pock extracts to remove aggregates of
virus eventually results in the selection of particular genotypes. These are then stable as judged by several passages at high multiplicity followed by successive single pock pickings. While a single white pock may, even after several cycles of single pock picking, give rise to individual white subclones with different sizes of deletion and of duplicated sequence, indistinguishable genotypes have arisen independently from separate, parental red clones. Heterogeneity among the progeny of a single white pock does not seem to result from inadequate pock purification, as red parental contaminants are lost easily, but rather from the propagation of a clone during the generation of the chorioallantoic lesion at subsequent passage. Eventually, certain genotypic configurations, incapable of further variation, are selected randomly. Some homogeneous clones have been derived easily at the first or second pock picking while other isolates remain heterogeneous after many cycles of pock picking.

The mechanism by which such mutant genome structures are generated is not clear but there are at least two possibilities. Firstly, that a terminal deletion is compensated by sequences copied from those mapping at the opposite terminus implying conjunction of termini or, secondly, that reiterated sequences are acquired by recombination between similar molecules in head-to-tail configuration. A recombinational model has been proposed and requires the presence of multiple sets of repetitive sequences at internal sites (Moyer et al., 1980). However, in the analogous case of intertypic recombination between herpes simplex virus types 1 and 2, participants exchange topographically and functionally equivalent regions of the genome in a head-to-head, tail-to-tail alignment. Alternatively, replacement of deleted terminal sequences with a copy of sequences from the opposite terminus may result from the mechanism of DNA replication in a similar manner to the generation of the structurally analogous adenovirus defectives described by Daniell (1976). Adenovirus DNA replication proceeds by displacement of a single strand during the copying of the complementary parental strand. Inverted terminal repetitions permit annealing of the ends of the displaced strand to form a single-stranded circle with a 'panhandle', the duplex region of which presents a DNA replication initiation site identical to that of the parental duplex (Lechner & Kelly, 1977). In the event of a break in the displaced single strand, the truncated end may offer a primer for extension using the opposite end of the same strand as template. Thus, sequences from one end of the genome, including any aberration there, may be copied to the opposite terminus in a process involving conjunction of ends. Vaccinia virus DNA forms such single-stranded circles with a duplex stem after removal of the terminal crosslinks in vitro (Garon et al., 1978) and the removal of the terminal crosslinks in vivo is an early event in replication (Pogo, 1980). Wittek & Moss (1980) described the presence of multiple tandem copies of a 70 base pair (bp) repeat within the inverted terminal repetition of vaccinia virus DNA and proposed that these accelerate the self-annealing of displaced single strands to provide an initiation site for DNA replication. A molecular clone of the 70 (bp) vaccinia tandem repeat sequence hybridizes to the terminal restriction fragments of other orthopoxviruses, including cowpox, which thus contain a related sequence. Cowpox DNA terminal fragments, re-cleaved with *Hin*II and end-labelled using the Klenow fragment of DNA polymerase I, show a highly repetitive sequence that hybridizes with the cloned vaccinia 70 bp tandem repeat (L. Archard & R. Wittek, data not shown). We suggest that these orthopoxvirus mutants result from an adenovirus-like replication mechanism and that, for this reason, symmetrical termini are preserved in all cases. The predilection for deletion at a specific locus in cowpox virus DNA may result from a sequence or structure peculiarity at that point. This is being investigated by a comparative analysis of the unique junction fragments derived from a number of cowpox white pock variants.

REFERENCES


(Received 20 October 1983)