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Arginine deprivation and the generation of white variants in cowpox virus-infected cell cultures

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SUMMARY

The white pock variant of cowpox virus shows limited growth in chick embryo fibroblasts maintained in arginine-deprived culture medium. Since these conditions inhibit the growth of parental virus, there is a marked increase in the frequency of the white variant in the virus population recovered after passage in the absence of arginine. The variants generated in this system have been characterized by restriction endonuclease analysis of virus DNA in the total DNA recovered from infected cell cultures. Such analysis shows that the white variants arise as deletion mutants of the parental virus, but there was considerable heterogeneity in the restriction patterns of different isolates examined shortly after their generation. Further passage selected white cowpox virus populations with a stable genome configuration comparable with the DNA of pock-purified white variants.

INTRODUCTION

Most avian and mammalian poxviruses can grow on the chick chorioallantoic membrane with the production of characteristic lesions (Downie & Dumbell, 1956), and in the case of cowpox virus these virus-induced lesions, or pocks, are typically haemorrhagic (Downie, 1939). However, both passaged and primary cowpox virus isolates also produce white pocks at a frequency of about 1% in an otherwise haemorrhagic population (Downie & Haddock, 1952; van Tongeren, 1952). Isolated variants breed true, but repeated sub-culture from single red pocks consistently produces white variants on the chorioallantois. Extracts of chorioallantoic membranes infected with white cowpox virus lack the 'd' antigen which is demonstrable in precipitation reactions between red cowpox soluble antigens and hyperimmune antiserum (Rondle & Dumbell, 1962; Baxby & Rondle, 1968). These biological properties suggest that the cowpox white variant is a deletion mutant, and the analysis of virion DNA by cleavage with restriction endonucleases has shown indeed that the white pock variants are produced by terminal deletions from the parental genome (Archard & Mackett, 1979).

Although the nature of the relationship between red cowpox and its white variant has been established, the mechanism which generates the deletion mutant

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remains unknown. In this paper withdrawal of the amino acid arginine from medium supplied to cowpox virus-infected cell cultures is shown to result in progeny virus populations with an increased frequency of the white variants. A number of these variants have been examined by restriction enzyme analysis of virus-specific DNA present in the total DNA isolated from infected cultures rather than from purified virus particles. Such modification permits the genotypic analysis of virus recovered from single pocks without further, extensive replication. This study describes the genomic properties revealed by cleavage with restriction endonucleases *Hind III* and *Xho I* of cowpox white variants immediately following their generation and after further passage in cell culture.

MATERIALS AND METHODS

Virus

The Brighton strain of cowpox virus was propagated by growth on the chorioallantois of 12–day fertile hen's eggs. To prepare the virus stock used in these studies an isolated, haemorrhagic pock was excised from the chorioallantoic membrane and dispersed mechanically into 5 mM-citrate–phosphate (McIlvaine's) buffer pH 7·4, before further disruption by treatment in an ultrasonic bath (Megasonic 80 kc) for 2 min. Following clarification by centrifugation at 1500 *g* for 5 min the suspension of recovered virus was inoculated onto the chorioallantois at a dilution calculated to give confluent infection. After incubation at 36 °C for three days the infected membranes were removed, and purified virus was prepared essentially as described by Joklik (1962).

Infectivity titrations were made either by pock formation on the chorioallantoic membrane or by plaque assays in confluent monolayers of Vero cell cultures.

Primary cell culture

Chick embryo fibroblasts (CEF) were prepared from the skeletal muscle of 10-day embryos by treatment of tissue fragments with 0·1 % trypsin in PBS at 37 °C for 30 min. The cells recovered were grown in Eagle's minimum essential medium (Eagle, 1959) supplemented with 10 % (v/v) calf serum, and confluent cultures were maintained in the same defined medium with 2 % (v/v) serum. Depletion of intracellular pools of arginine was achieved by incubation of cell monolayers for 18 h in arginine-free maintenance medium.

DNA extraction

CEF cultures (approximately 10^7 cells) were infected and maintained in medium supplemented with 0·5 μCi/ml [6-^{3}H]thymidine (23 Ci/mmol) until the virus cytopathic effect observed microscopically was seen to involve more than 80 % of the cell population. At this stage the infected cells were recovered with 0·02 % EDTA in PBS and the cell suspension centrifuged at 500 *g* for 5 min. The infected cells were resuspended in 50 mM-tris-HCl, pH 7·8, 1 mM-EDTA and 30 % sucrose before lysis at 4 °C by the addition of 1 % sodium *n*-lauryl sarcosinate and 100 mM-mercaptoethanol. After 30 min proteinase K was added to a final concentration of 500 μg/ml and further digestion continued at 37 °C for 2 h. Solutions were extracted with buffer-saturated phenol and finally with chloroform:isoamyl

alcohol (24:1) to remove protein before precipitation of DNA at −20 °C with 2·5 vol. ethanol. The DNA recovered was redissolved in 10 mM-tris-HCl, pH 7·5, 1 mM-EDTA.

Restriction enzyme analysis

DNA was cleaved with restriction endonucleases *Hind III* or *Xho I* and restriction fragments were separated electrophoretically in 0·6% agarose as described previously (Archard & Mackett, 1979). Gels were treated for 20 min with 1 M-sodium salicylate, dried and radioactively-labelled DNA was visualized by autoradiography at −70 °C using pre-sensitized Fuji Rx X-ray film.

RESULTS

Arginine deprivation and virus growth

The replication of cowpox virus in CEF cultures was examined both in the presence and in the absence of arginine. Intracellular pools of arginine were depleted as described before cell cultures were infected at a multiplicity of 0·1 plaque-forming units/cell. After an adsorption period of 1 h (time zero), the monolayers were washed with maintenance medium to remove unadsorbed virus before the infected cultures were maintained further, either in arginine-deprived medium or in medium containing 1·0 mM-arginine. Samples from each series were taken at various times after infection, and titrations of total infective virus were made by plaque formation (Table 1). In complete medium there was a significant virus yield at 12 h post-infection with maximum titres 36 h later, but virus growth in the absence of arginine was markedly suppressed even after incubation of infected cultures for a total of 48 h.

Arginine deprivation and the generation of white pock variants

In addition to the assays described above, parallel infectivity titrations were made by pock formation on the chorioallantoic membrane (Table 2). Such titrations confirmed the inhibitory effect of arginine deprivation on the replication of cowpox virus in CEF cultures. However, the additional facility of phenotypic characterization shows that the proportion of white pock variants in the infective, progeny virus is determined by the availability of arginine. The virus populations recovered from infected cultures immediately after the adsorption period produced predominantly haemorrhagic pocks with only 1% white pock variants: these proportions were unchanged following virus replication in the presence of arginine. Although the withdrawal of arginine resulted in a marked reduction in virus yield at 48 h post-infection, there was a sevenfold increase in the proportion of the white pock phenotype.

This increased frequency of white pock variants generated by passage of cowpox virus in CEF cultures maintained in arginine-free medium suggested that such conditions may act selectively on virus growth. Consequently, a pock-purified preparation of the cowpox white variant was used to infect CEF cultures which were maintained subsequently in appropriate media. With an inoculum recovery of $4·7 \times 10^3$ plaque-forming units (p.f.u.)/ml, the infectivity titres measured at 24 h post-infection were $5·5 \times 10^5$ p.f.u./ml with complete medium and $5·6 \times 10^4$

Table 1. *Effect of arginine deprivation on the growth of cowpox virus in chick embryo fibroblasts*

Time (h p.i.)	Infectivity titre* (plaque-forming units/ml)	
	Complete medium	Arginine-deprived medium
0	$5{\cdot}8 \times 10^3$	$5{\cdot}8 \times 10^3$
12	$4{\cdot}0 \times 10^4$	$7{\cdot}2 \times 10^3$
24	$2{\cdot}1 \times 10^5$	$9{\cdot}1 \times 10^3$
48	$6{\cdot}6 \times 10^6$	$1{\cdot}2 \times 10^4$

* Infectivity titrations were made by plaque assay in confluent monolayers of Vero cells.

Table 2. *Effect of arginine deprivation on the generation of white pock variants of cowpox virus*

Medium	Infectivity titre (pock-forming units/ml)*		
	Red pocks	White pocks	Total
Complete (time zero)	$8{\cdot}6 \times 10^3$	$1{\cdot}0 \times 10^3$ (1·1 %)	$8{\cdot}7 \times 10^3$
Complete (48 h p.i.)	$6{\cdot}3 \times 10^6$	$8{\cdot}0 \times 10^4$ (1·2 %)	$6{\cdot}4 \times 10^6$
Arginine-deprived (48 h p.i.)	$1{\cdot}5 \times 10^4$	$1{\cdot}2 \times 10^3$ (7·5 %)	$1{\cdot}6 \times 10^4$

* A minimum of 1000 pocks were counted at 72 h p.i. on chorioallantoic membranes inoculated with virus dilutions which produced discrete lesions.

p.f.u./ml with arginine-deprived medium. These results show that, unlike parental virus, limited replication of the white pock variant can occur in the absence of arginine.

Isolation of white pock variants generated by arginine deprivation

After the third successive passage in the absence of arginine, nearly 40 % of the infective virus recovered from cowpox-infected CEF cultures had the white phenotype. At this stage inoculation onto the chorioallantois readily produced infected membranes with white pocks only. Single pocks were excised from such membranes to give eight separate isolates, and each pock was disrupted to release virus as described previously. Infectivity titrations showed that there was a wide variation in the amount of infective virus recovered from each pock (Fig. 1). The titres obtained with isolates 3 and 4 were 1000-fold less than the values for isolates 7 and 8. Intermediate titres were obtained with the other isolates apart from isolate 1, which did not contain infective virus detectable by either plaque or pock assay.

Restriction enzyme analysis of white pock variants

Lonsdale (1979) has shown that virus-specific patterns can be recognized readily after electrophoretic separation of restriction enzyme digests of total DNA recovered from herpes simplex virus-infected cell cultures. Such discrimination is based on low levels of cellular DNA synthesis in confluent monolayers of primary cell cultures, which results in the preferential incorporation of radiolabelled

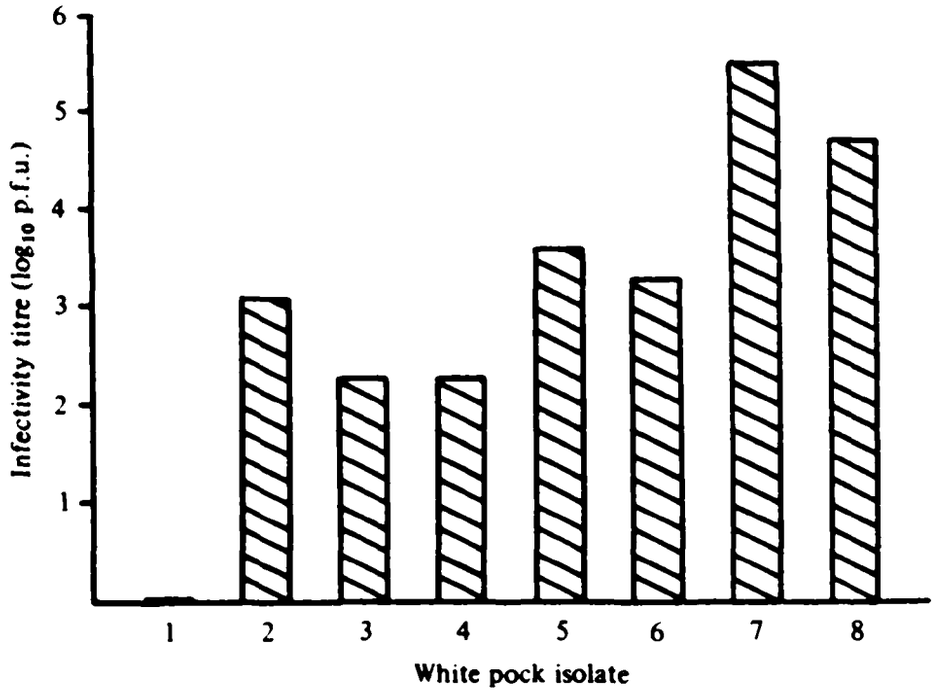

Fig. 1. Amount of infective virus recovered from the lesion produced on the chorioallantoic membrane by white pock variants generated by passage of cowpox virus in arginine-deprived chick embryo fibroblasts.

precursors into virus DNA following infection. Subsequent autoradiographic examination of the electrophoretograms reveals the virus-specific DNA fragments. This analytical technique has been used in the present study to characterize virus DNA in total DNA extracts from cowpox virus-infected CEF cultures.

Preliminary experiments were made to compare red cowpox virus with its white variant produced by direct inoculation of the cowpox virus stock onto the chorioallantoic membrane. Virus recovered from isolated lesions of the appropriate phenotype was pock-purified by two further passages at high dilution before the extract from a single red or white pock was inoculated onto confluent CEF cultures. The DNA recovered from infected cells was cleaved with *Hind III* or *Xho I* and the fragments visualized are identified alphabetically in order of decreasing molecular weight (Plate 1 a, b). Comparison of the restriction patterns shows that many fragments from each DNA preparation co-migrated electrophoresis but fragments B, H and L obtained by *Hind III* digestion of DNA from cultures infected with red cowpox virus were absent from similar digests prepared from cultures infected with the white pock variant. Similarly, fragments E, L and O produced by *Xho I* digestion were unique to the parental virus. Such distinctions are entirely consistent with the results reported previously with virus DNA prepared from purified virions (Archard & Mackett, 1979). Specific patterns were not resolved with control preparations from uninfected cells apart from one repetitive sequence of host cell DNA produced by *Hind III* digestion (Plate 1 a). These preliminary studies show virus-specific fragments can be visualized by the techniques described after digestion with endonucleases *Hind III* or *Xho I* of the total DNA recovered from cowpox virus-infected CEF cultures.

Similar methods were used to characterize the white pock variants generated by passage of cowpox virus in arginine-deprived cell cultures. Virus recovered from

each pock was inoculated directly onto confluent CEF monolayers, which were maintained in complete medium until extensive cytopathic effects had appeared before total DNA was recovered and digested with appropriate restriction enzymes. After electrophoretic separation a wide variety of restriction patterns was obtained with the different isolates (Plate 1 c, d). In view of the failure to recover infective virus, it is interesting that virus-specific restriction patterns were obtained with pock isolate 1. In general, the *Hind III* and *Xho I* fragments unique to red cowpox virus DNA were not detected, but other restriction fragments were also absent from several isolates, particularly isolates 1, 3 and 4. Further comparison shows restriction fragments which do not co-migrate with the restriction products obtained from the total DNA of red cowpox virus-infected cultures, for example fragment X obtained with *Hind III* digests. This qualitative heterogeneity between different isolates was accompanied by other, quantitative discrepancies. Since the amount of radioactivity present will be related directly to the molecular weight of each restriction fragment, there should be an inverse relationship between electrophoretic mobility and the density of silver grains produced after autoradiography. However, the intensity of development of some restriction fragments was less than their molecular weights would require, which indicates that several isolates, for example isolate 4, contain heterogeneous populations of virus genomes.

In order to examine the effect of further virus growth on the genotypic character of the white pock variants generated by arginine deprivation, isolates 4 and 5 were passaged twice in CEF cultures before the extraction of total DNA from infected cells was repeated. After cleavage with restriction endonuclease *Hind III* or *Xho I* the fragment pattern obtained with passaged isolate 4 showed both qualitative and quantitative identity with passaged isolate 5. Similar results were obtained by passage in CEF cultures maintained either in the presence or in the absence of arginine. The restriction pattern of each passaged isolate was essentially indistinguishable from those obtained with the pock-purified preparations of white cowpox virus (Plate 1 a, b).

DISCUSSION

The requirement for arginine in the growth of cowpox virus demonstrated in the present study is exhibited also during the replication of vaccinia virus (Holtermann, 1969) and of rabbitpox virus (Cooke & Williamson, 1973). Withdrawal of the essential amino acid from culture medium supplied to infected cells resulted in complete inhibition of the production of infectious progeny virus. Unlike parental virus, however, limited growth of the cowpox white variant still occurred under conditions of arginine deprivation. Arginine biosynthesis from citrulline mediated by virus-specific enzymes has been shown to be induced in vaccinia or rabbitpox-infected cells but growth of both viruses is inhibited in the absence of the metabolic precursor (Cooke & Williamson, 1973; Williamson & Cooke, 1973). In the absence of *de novo* synthesis arginine deprivation of continuous cell lines has been shown to increase protein turnover, resulting in replenished amino acid pools (Eagle & Levintow, 1965). Similar metabolic changes in cowpox virus-infected chick embryo cultures maintained in deprived medium must provide arginine under conditions that permit growth of the white variant but not parental virus.

Although the replication mechanism which generates the cowpox virus variants remains unknown, their selected growth together with a modified method of restriction enzyme analysis has permitted qualitative examination of virus DNA at an earlier stage in the process than had been possible before. The white pock variants examined in previous studies had been subject to extensive serial passage before isolation of virion DNA (Archard & Mackett, 1979; Mackett & Archard, 1979) but genotypic characterization has been achieved in the present study after limited virus replication. In addition, total virus-specific DNA synthesized in infected cells has been characterized rather than DNA recovered only from purified virus particles. These modified techniques have revealed considerable heterogeneity in the restriction patterns of different white pock isolates made shortly after their generation. At this stage it appears also that a heterogeneous virus population may be recovered from single lesions on the infected chorioallantoic membrane. Examination of the restriction patterns confirms that the white variants are deletion mutants of red cowpox virus, a relationship demonstrated earlier by construction of physical maps of the virus genomes (Archard & Mackett, 1979). By comparison with these maps the genomes of some white variants generated by passage in arginine-deprived CEF cultures lack sequences unique to the red parent which are found at the right-hand terminus of the virus genome. Other variants, however, appeared to have additional deletions from the left-hand terminus, and these pock isolates contained the lowest amounts of infective virus. White pock mutants of monkeypox virus characterized by DNA analysis have similar classes of terminal deletions (Dumbell & Archard, 1980).

Two of the cowpox white pock variants isolated in the present study were distinguishable by restriction enzyme analysis on initial isolation but became genotypically homogeneous after further passage. Qualitative characterization with restriction endonucleases *Hind III* and *Xho I* showed their identity with virus DNA recovered from pock-purified variants. This genome structure, which had deletions from the right-hand terminus only, does not change after further replication and it appears, therefore to represent a stable configuration. The same restriction patterns were obtained after further growth of these white pock isolates in CEF cultures maintained in complete medium or in the absence of arginine. This suggests that arginine deprivation exerts a selective pressure on the growth of the different variants at a post-replicative stage in the virus growth cycle. Certain deletion mutants of rabbitpox virus isolated as white variants are unable to grow in cell systems which are permissive to other mutants of a similar phenotype (Moyer & Rothe, 1980). A similar mechanism may determine the nature of the deletion mutants of cowpox virus obtained after extended passage. Although it is not known how this stable cowpox mutant is able to replicate in the absence of arginine, it is interesting that this facility provides a selective advantage over the parental virus.

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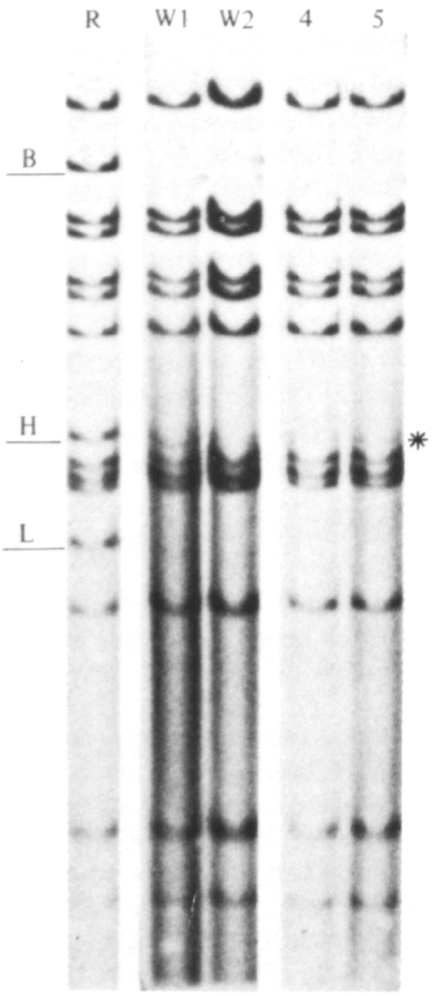

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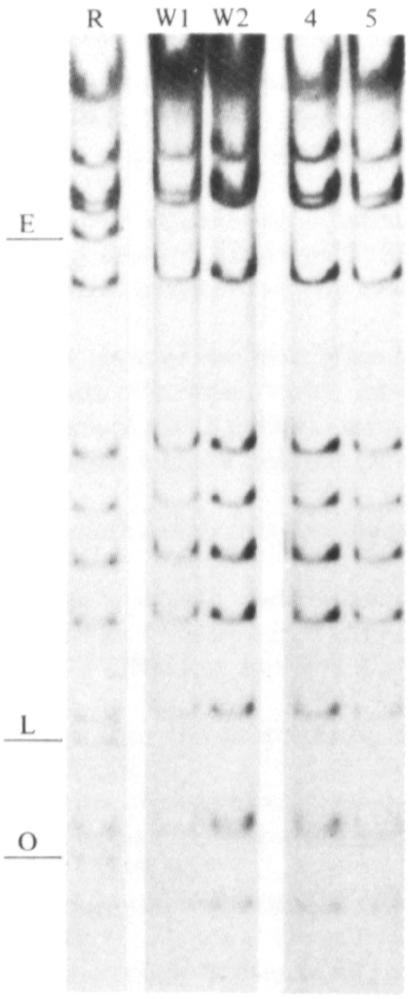

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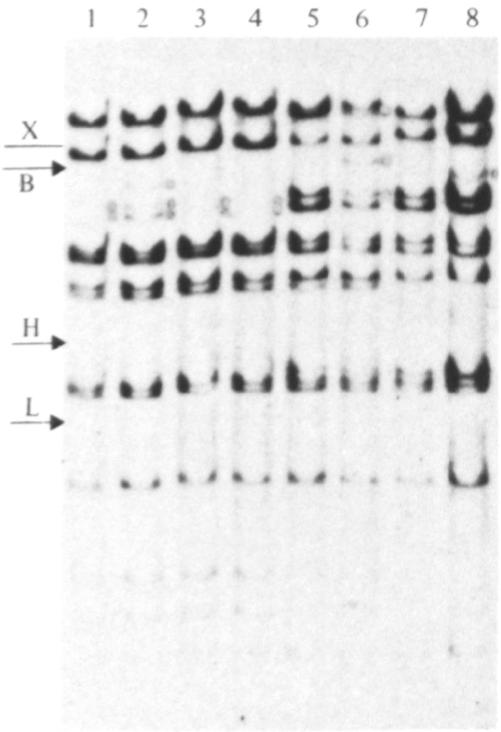

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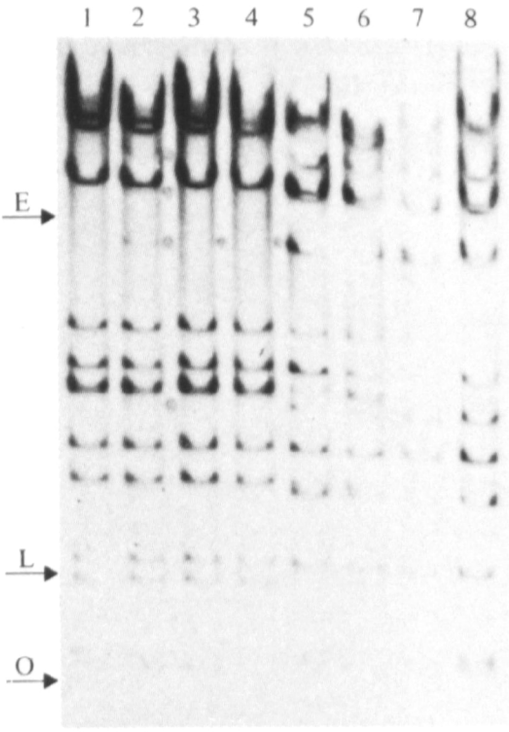

(*d*)

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EXPLANATION OF PLATE

Plate 1. (*a*) ***Hind III***, (*b*) ***Xho I*** restriction fragments of DNA from chick embryo fibroblasts infected with red cowpox virus (R); pock-purified white cowpox virus (W1 and W2); and white pock variants (isolates 4 and 5) after extended passage in arginine-deprived chick fibroblast cultures. Restriction fragments unique to red cowpox virus are lettered and an asterisk marks the ***Hind III*** fragment derived from repetitive sequences in host cell DNA.

(*c*) ***Hind III***, (*d*) ***Xho I*** restriction fragments of DNA from chick embryo fibroblasts infected while pock variants (isolates 1 to 8) generated by limited passage of cowpox virus in arginine-deprived chick fibroblast cultures. The arrows mark the distance of migrations of restriction fragments unique to red cowpox virus. The ***Hind III*** fragment X is an example of a restriction fragment which does not co-migrate with the restriction products obtained from the total DNA of red cowpox virus-infected cultures.

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