Correction. In the article "The \( r \)-Cation Radical of Chlorophyll \( a \)," by Donald C. Borg, Jack Fajer, Ronald H. Felton, and David Dolphin, which appeared in the October 1970 issue of *Proc. Nat. Acad. Sci. USA*, 67, 813–820, an editorial change in the mode of abbreviation led to an error on page 818, line 6. For ZnPh4P\(+\), read ZnPh4C\(+\). (the cation radical of zinc tetraphenylchlorin). The sentence should read as follows: "Other possibilities such as specific oxidation of a substituent group of Chl \( a \) are ruled out by the above argument and by the close similarity of the electronic spectra of Chl\(+\) and ZnPh4C\(+\). (the cation radical of zinc tetraphenylchlorin)."


Correction. In the article "Revised Identification of the Chromophore of a Cell Division Factor from Crown Gall Tumor Cells of *Vinca rosea* L.," by Henry N. Wood, which appeared in the November 1970 issue of *Proc. Nat. Acad. Sci. USA*, 67, 1283–1287, the following corrections should be made:

(a) Page 1284, line 6 from bottom: 0.85, not 9.85.

(b) Page 1284, line 2 from bottom: insert "272," between "279," and "199."

(c) Page 1287, add to explanation of "Abbreviation" the words "in a butanol–water system after 500 transfers in countercurrent distribution."
Nuclear Involvement Poxvirus Infection

KIRSTEN H. WALEN*

School of Biological Sciences, The University of Sydney, Sydney, NSW2006 Australia

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ABSTRACT During the first hours of infection with vaccinia virus, part of the chromosomal DNA of host cells is degraded, part of it is released from the cell, and part of it eventually appears in cytoplasmic foci associated with the reproduction of virus. The DNA of the infecting particle is first found attached to the chromosomes. Intracellular foci of DNA incorporation appear later; these rapidly move out into the perinuclear space and eventually become typical cytoplasmic “virus factories.” Each of these observations points to a more direct involvement of the nucleus in viral infection than was previously envisaged.

On entering a susceptible cell, vaccinia virus undergoes a two-stage uncoating process (1) and eventually multiplies in cytoplasmic foci called “virus factories” (2) which are easily demonstrated with autoradiography and nuclear stains. Even though early autoradiographic (2) and biochemical studies hinted at no nuclear involvement, viral DNA is not produced in cells treated before infection with mitomycin C (3), nor in leukocytes incapable of synthesizing DNA (4). In fact, there is a dark period between uncoating [when coreless shells remain in the cytoplasm and the DNA-containing cores disappear from view (5)] and the establishment of virus factories some hours later.

My experiments are concerned with this dark period and suggest that nuclear DNA is broken down during viral replication; that some of this DNA becomes part of the viral factories; and that the factories themselves first appear in close proximity to chromosomal material, only to move later into the cytoplasm. More detailed accounts will be published elsewhere.

MATERIALS AND METHODS

Vaccinia virus (strain VMH or WR†) was grown in coverslip cultures of African green monkey cells (BS-C-1), maintained in Eagle’s medium plus 10% fetal-calf serum. Confluent monolayers of host tissue were covered with inocula and thoroughly washed at the end of a 1-hr adsorption period. The course of infection was followed by pulse-labeling with [3H]-thymidine (3000 Ci/mol, applied as 0.01 ml of a dilution containing 1-2 µCi). The slips were fixed in Carnoy’s fluid, stained with either aceto-orcein, which stains virus factories, or Wright’s stain, and autoradiographed on Kodak NTB liquid emulsion no. 2. The processed slips were mounted in Euparal.

RESULTS

The validity of the test criterion, incorporation of tritiated thymidine into cytoplasmic inclusions, was established. In a preliminary experiment, dilutions of WR virus were mixed with specific anti-vaccinia serum and 0.01 ml of the mixtures was applied to two coverslips each. After a 4-hr incubation, the slips were layered with 0.01 ml of medium containing [3H]thymidine for 15 min, washed, and immediately processed. The number of cells with virus factories was reduced by more than 90% at the two multiplicities of infection tested, at 50 PFU/cell, from 484 to 43 cells, and at 5 PFU/cell, from 52 to 4 cells. It is concluded that antiserum that neutralizes viral infectivity also prevents appearance of cytoplasmic foci of thymidine incorporation.

Incorporation of thymidine during the early stages of infection

Cells shown by autoradiography to be mostly in the S period were infected at a multiplicity of 10 PFU/cell, and pulse labeled with [3H]thymidine for 15 min at 0.5-hr intervals. The tissues were fixed either immediately after floating with cold thymidine or at 4-hr after infection. Virus factories ap-

<table>
<thead>
<tr>
<th>Hr after infection: of [3H]thymidine pulse</th>
<th>1–1.5</th>
<th>1.5–2</th>
<th>2.25</th>
<th>2.5–3</th>
</tr>
</thead>
<tbody>
<tr>
<td>of harvest times</td>
<td>1.5</td>
<td>2</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. of cells with factories</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>No. of nuclei labeled</td>
<td>754</td>
<td>718</td>
<td>718</td>
<td>675</td>
</tr>
<tr>
<td>No. of nuclei labeled</td>
<td>652</td>
<td>653</td>
<td>644</td>
<td>608</td>
</tr>
<tr>
<td>Expt. 2</td>
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<td></td>
</tr>
<tr>
<td>No. of cells with factories</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>No. of nuclei labeled</td>
<td>543</td>
<td>521</td>
<td>555</td>
<td>512</td>
</tr>
<tr>
<td>No. of nuclei labeled</td>
<td>508</td>
<td>486</td>
<td>473</td>
<td>438</td>
</tr>
</tbody>
</table>

* Numbers are averages of 4 counts across the median of 2 coverslips.

Abbreviation: PFU, plaque-forming units.

* This work was started during tenure of a U.S. Public Health Service Special Fellowship (1-F3-GM-32,355-01) in the Department of Microbiology at the Australian National University, Canberra, A.C.T. In the present location the project was supported by the University of Sydney Research Grants Committee and by the Australian Research Grants Committee.

† Vaccinia virus strains VMH and WR with titers of plaque units of 1 × 10⁶ and 5 × 10⁶, respectively, were kindly supplied by Dr. F. Fenner.
The presence of labeled virus factories was fully developed (Fig. 1c). Uninfected cells (Fig. 1a) had the typical background of only a few isolated grains.

That the labeled debris was degraded DNA was demonstrated more clearly as follows. Monolayers of BS-C-1 cells were infected and pulse-labeled after 2 hr. Some cultures were fixed 30-min after labeling (set A), the rest seeded with a layer of morphologically distinguishable cells (chick-embryo fibroblasts or a line of stable marsupial cells†). After 1 hr, these cultures were again divided; half of them received a second pulse of [3H]thymidine (set B), the other half (set C) were untreated. Samples of sets B and C were fixed at 0.5-hr intervals for up to 5-hr of infection. A series of uninfected control slides was carried in parallel through the same procedure.

Harvest A and B verified the labeling of the primary and secondary monolayers respectively, with results entirely comparable to those shown in Fig. 1. While the secondary cells over uninfected controls were uniformly blank, both the chick and marsupial cells plated over infected primary layers (set C) showed light but consistent nuclear labeling of 1–16 grains.

**Fate of nuclear DNA during viral replication**

The extensive release of nuclear DNA fragments soon after infection raised the possibility that this material might serve as substrate during subsequent replication of viral DNA. Host cells were therefore labeled 48 and/or 2 hr before infection, then autoradiographed 4-hr after infection.

The atypical background of labeled debris was present in all infected cultures (Fig. 2a), but could be greatly reduced by extraction with cold CH3C00H. Nuclear labeling was reduced in the infected series (Fig. 2b), with often irregular distribution of grains over the nuclei. Labeled cytoplasmic inclusions (Fig. 2c) were found only in cells with labeled nuclei, but even in such cells not all virus factories were labeled. On the whole, the heavier the nuclei were labeled, the higher the proportion of labeled viral foci. At least some of the viral DNA is thus seen to originate from the chromosomal material of the host cell.

**The source of viral DNA**

The presence of labeled as well as unlabeled virus factories in the same cytoplasm militates against the free availability of nuclear breakdown products. The next experiment therefore tested whether the virus reacted in some direct manner with the chromosomes of its host.

Partially synchronized cultures were infected in the G2 period (i.e., after synthesis of nuclear DNA was completed), and, after treatment with colchicine, were collected in prophase or metaphase. At various times after infection the cultures were pulse-labeled and fixed immediately after the labeling period. As expected uninfected control cells showed no labeling of mitotic chromosomes, since the G2 period lasts 4.5–5 hr in BS-C-1 cells. Infected cells, on the other hand, had foci of radioactivity associated with highly damaged chromosomes. These familiar types of chromosomal ab-

† Secondary chick embryo fibroblasts and *Potorous tridactylus* kidney (PtK-1) cells.

normalities ranged from stickiness and uncoiling to complete disintegration and are best demonstrated without silver grains (Fig. 3a and b).

**Association of viral DNA and host chromosomes**

To exclude unwanted side effects due to colchicine, and possible complications arising from the use of replicating virus, the following experiment was designed. Cells, predominantly in the G2 period, were infected with heavily-labeled virus that had been allowed to "suicide" during storage over the past 8 years.§ The cells were fixed after 3 hr of infection. As the micrographs of Fig. 4 show, the DNA of the killed virus particles was associated with the metaphase and anaphase figures. The density of grains suggests single viral genomes rather than foci of replication, such as are found after infection with live virus. Infection of G1, S, or G2 cells with the dead virus did not result in either cytoplasmic foci or labeled nuclei.

**Migration of replicative foci in the course of infection**

The last two experiments demonstrated association between viral DNA and the nuclear apparatus of the host cell, whereas virus factories have hitherto been seen only in the cytoplasm.

To bridge this gap, use was made of cells predominantly in the G1 period; the cells themselves do not incorporate thymidine during this period of the mitotic cycle, therefore events in the nuclear space can be more readily followed.

Unlabeled BS-C-1 cells, mostly in G1, were infected at a multiplicity of 10; several cultures were pulse-labeled at 0.5 hr intervals and fixed directly after labeling. In these cultures (Fig. 5) the few S-phase cells served as the 0-hr control. It is obvious that at 2.5 hr, [3H]thymidine is incorporated differently than the earlier intervals when the label is confined to the nucleus. From 3-hr after infection, only the typical cytoplasmic virus factories are to be seen.

**DISCUSSION**

Hints of nuclear involvement in the process of poxvirus infection abound in the literature. Some (1, 3, 4) imply the need for an early host function, while others (6) describe the loss of about 10% of the total cellular DNA that is not accounted for by an increase in the acid-soluble pool. My observations confirm the degradation of DNA and fix the temporal position of the process. Labeled debris is released within 2 hr of infection, and thus invariably before the appearance of cytoplasmic viral factories. The negative aspect of the same event is the striking loss of label from previously labeled nuclei (Fig. 2), the increasingly abnormal appearance of the chromosomes (Fig. 3), and the reduced incorporation of thymidine into the nuclear apparatus after the 2nd hour of infection (Table 1). These are all direct observations, and the only point to be settled is whether or not they represent a regular stage in the infective process. For example, breakdown of cellular DNA could well be caused by *Mycoplasma* (7), a subtle and frequent contaminant of tissue cultures. I have encountered *Mycoplasma* twice in these experiments but, unlike the situation in biochemical work, its presence is readily detected both in cytological and autoradiographic preparations by distinct cytopathology and abnormal labeling features. Other chance contaminants are even more obvious under the microscope.

§ Dr. J. Cairns labeled and purified vaccinia virus in 1962–63. This virus was stored at −70°C until it was obtained through the courtesy of Dr. F. Fenner for the present experiments.
Fig. 1. Background in autoradiographs of control (a) and infected (b and c) cells.

Fig. 2. Cells labeled before infection: (a) atypical background, (b) abnormal distribution of grains over the nuclei, and (c) labeled viral factories.

Fig. 3. Cells infected in G1 and collected in mitosis with colchicine treatment: (a) sticky metaphase chromosomes (above) adjacent to normal appearing chromosomes, and (b) viral factories (dense bodies) associated with disintegrated chromosomes.

Fig. 4. Silver grains from labeled virus over metaphase (a) and anaphase (b) chromosomes.

Fig. 5. Labeling of infected G1 cells at various times after infection.
The evidence goes further than merely demonstrating nuclear breakdown and incorporation of some of its products in what have become accepted as cytoplasmic virus factories (8). At the time label is lost from the chromosomes, aggregations of DNA-containing material appear within the nucleus (presumably associated with the chromosomes if one may extrapolate from observations with killed virus as shown in Fig. 4). Morphologically, these foci are not distinguishable from the virus factories to be found in the cytoplasm about 0.5-hr later. Indeed, the sequence presented in Fig. 5 suggests that the nuclear foci are precursors of the cytoplasmic foci.

The nuclear residence of these foci is short, probably less than 0.5-hr. Since the course of infection is asynchronous (8), biochemical assays representing the average behavior of perhaps millions of cells are bound to miss an event of such brief duration. Autoradiography is better suited to resolve the intracellular sequence, provided the cells are not moving randomly through their mitotic cycles. If even a small fraction of the usually long S-period coincides with the intranuclear stage of viral replication, the heavily-labeled chromosomes will completely obscure the viral foci. Hence, only multiple infection followed by short pulses of labeling over the G1 or G2 periods offers any hope of demonstrating this phase.

All aspects of this work, including revision of the manuscript, were greatly improved by discussions with Dr. S. Fazekas de St. Groth. I am also deeply grateful to Dr. S. W. Brown for evaluation of the cytological aspects and for continuing encouragement. The photographic assistance of Messrs. B. Lester and J. Fairburn is also gratefully acknowledged. The final draft of the manuscript was prepared in the Department of Genetics, University of California, Berkeley.