Multiple Protein Functions in the Replication of Pox Virus DNA

(Accepted 11 March 1968)

The replication of pox virus DNA is known to require the prior synthesis of new proteins. It can thus be completely inhibited by the addition early after infection of either puromycin (Joklik & Becker, 1964) or p-fluorophenylalanine (FPA) (Appleyard & Zwartouw, 1965). This dependence upon protein synthesis has been related to a requirement for new enzymes and particularly the DNA polymerase which has been demonstrated in pox virus-infected cells (Magee, 1962; Green & Piña, 1962; Jungwirth & Joklik, 1965). Recently, however, Kates & McAuslan (1967) suggested that there is also need for a second type of protein function stoichiometrically related to DNA synthesis. We here draw attention to a difference in the action of puromycin and FPA which supports this interpretation and record additional findings which suggest that a third protein function may be needed to make ‘functional’ virus DNA. The term ‘functional’ is here used for DNA which has been recognized and quantified by the development of infectious virus after further DNA synthesis has been blocked by the addition of either \(10^{-5.7}\) M-5-fluorodeoxyuridine (FUDR) or \(10^{-4.9}\) M-5-bromo-deoxyuridine (BUDR) (after Salzman, 1960).

Our findings principally concern HeLa cells infected with variola virus, although additional experiments have been made with vaccinia and rabbit pox viruses. One-step growth conditions were achieved by infection of monolayers in 6 cm. diam. Petri dishes with 10 to 20 pk. f.u. of virus/cell. After adsorption for 1 hr virus was removed and the cells incubated at 35°C in Eagle’s medium with 2% calf serum. Puromycin dihydrochloride (Nutritional Biochemicals Corporation) was used at a concentration of 15 μg./ml. DL-FPA (Koch-Light Laboratories Ltd.) was used either at 500 μg./ml. in the presence of phenylalanine 33 μg./ml., or at 100 μg./ml. in the presence of phenylalanine 6 μg./ml.; this variation did not affect the inhibition in any way. Virus titrations were made by poch counts in chick embryos. The formation of virus DNA was observed: (1) by the accumulation of ‘functional’ DNA, (2) by the development of cytoplasmic inclusions in cells stained with acridine orange (coverslip cultures), and (3) by the incorporation of \([\text{H}]\)thymidine into acid-insoluble material in the cytoplasm of cells fractionated by a technique which, in control experiments with labelled uninfected cells, broke over 99% of cells and gave a recovery of only 2 to 3% of the radioactivity in the cytoplasmic fraction.

Some basic features of the growth in HeLa cells of variola virus and its DNA are presented in Table 1. Also shown is the expected almost complete inhibition of synthesis of virus DNA produced by addition early after infection of either puromycin or FPA. This addition was mostly made at 2 hr which in this system is 2 hr before the first appearance of newly synthesized virus DNA and yet is probably after the occurrence of certain very early events which also require protein synthesis (Joklik, 1964). If the addition of anti-metabolite was delayed until later, 3 hr or more after infection, synthesis of virus DNA was no longer completely suppressed. At this stage
**Table 1.** The influence upon virus DNA synthesis and virus growth of puromycin and FPA added 1 or 2 hr after infection

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percentage cells positive (acridine orange)</th>
<th>Titrations of infectivity (pk.f.u./cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[(\text{H}\text{]thymidine incorporation}^*]</td>
<td>Functional DNA at 8 hr†</td>
</tr>
<tr>
<td>None</td>
<td>94-98</td>
<td>2685</td>
</tr>
<tr>
<td>FPA</td>
<td>0-1</td>
<td>151</td>
</tr>
<tr>
<td>Puromycin</td>
<td>0-4</td>
<td>49</td>
</tr>
</tbody>
</table>

* Counts/min. in cytoplasmic fraction (acid-insoluble material) after labelling for 30 min. with [\(\text{H}\text{]thymidine (1-0 \(\mu\)c/ml.; 3-0 c/mole).}

† 24 hr virus yield after addition of \(10^{-4}\^M\)-FUdR at 8 hr; when present FPA was first removed and the tissue cultures washed before adding fresh medium containing FUdR and a supplement of phenylalanine to a concentration of 100 \(\mu\)g./ml.

~, Not done.

---

Fig. 1. Replication of virus DNA in the period 4 to 8 hr after infection in the absence of inhibitors (●●●), in the presence of FPA added at 4 hr (■■■) and in the presence of puromycin added at 4 hr (▲▲▲). (a) Synthesis of DNA determined as radioactivity in cytoplasmic fraction after continuous labelling with [\(\text{H}\text{]thymidine (1-0 \(\mu\)c/ml.; 1-0 c/mole).}

(b) growth of functional DNA determined by virus yield at 24 hr after addition of \(10^{-4}\^M\)-BUdR at times plotted (FPA where present was first removed as described in footnote to Table 1).

we were able to demonstrate a difference in the response to FPA and to puromycin. This is shown in the experiment recorded in Fig. 1 in which FPA or puromycin was added 4 hr after infection. The synthesis of virus DNA was followed over the period 4 to 8 hr by continuous labelling with [\(\text{H}\text{]thymidine (Fig. 1 (a)) and in the presence of FPA it continued over the whole period and at a rate not greatly less than that of control uninhibited cultures. By contrast, synthesis in the presence of puromycin was much reduced and particularly so in the period 6 to 8 hr. The difference in response was shown even more clearly when FPA or puromycin was added 6 hr after infection and the rates of incorporation measured at 7 and 8 hr (Table 2). When DNA synthesis was followed in terms of ‘functional’ DNA (Fig. 1 (b)) it appeared that there was no detectable accumulation in the presence of FPA. This result was
surprising for [3H]thymidine incorporation had suggested a synthesis 65% of that in control cultures, whereas the level of ‘functional’ DNA did not rise significantly above the baseline and could not therefore have been more than 12 to 16% of that in controls. Nevertheless, this failure to obtain an increase in ‘functional’ DNA following delayed addition of FPA is something which we have observed repeatedly with variola virus. It is also in direct contrast to the findings of Appleyard & Zwartouw (1965) with rabbit pox virus and we therefore compared variola, vaccinia and rabbit pox viruses in this respect under our conditions (Fig. 2). Rabbit pox virus was indeed found to behave differently, whereas vaccinia, like variola, gave no detectable growth of ‘functional’ DNA after the addition of FPA. In a further experiment similar to that shown in Fig. 1 we confirmed that with vaccinia, as with variola, incorporation of [3H]thymidine into cytoplasmic DNA continued after the addition of FPA at 4 hr despite the absence of any increase in ‘functional’ DNA.

Table 2. The influence upon the subsequent rates of virus DNA synthesis of puromycin or FPA added 6 hr after infection

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>[3H]thymidine incorporation at times after infection*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hr</td>
</tr>
<tr>
<td>None</td>
<td></td>
</tr>
<tr>
<td>FPA</td>
<td>2145</td>
</tr>
<tr>
<td>Puromycin</td>
<td>2445</td>
</tr>
</tbody>
</table>

* See footnote to Table 1. — Not tested.

Much of the interest of these results stems from the varying susceptibility of different proteins to inhibition by FPA. This variation is inherent in the action of FPA and has been observed before in relation to different virus functions (e.g. Scholtissek & Rott, 1961; Levintow et al. 1962; Wecker, Hummeler & Goetz, 1962). These earlier studies were made with different RNA viruses, but show many features similar to those presented here. Puromycin, by contrast, may be regarded as a more universal inhibitor of protein synthesis. Although caution is always necessary in the interpretation of experiments involving metabolic inhibitors we believe that our results are most easily explained in terms of three protein functions associated with the replication of pox-virus DNA.

The first is the enzymic function which must be intrinsically sensitive to FPA, since FPA can inhibit its development, and yet must be stable as shown by its continued action in presence of FPA. This behaviour is quite in keeping with what is known of the synthesis and stability of the enzymes concerned in pox-virus DNA synthesis (Jungwirth & Joklik, 1965; Kates & McAuslan, 1967), although as yet this information relates only to the enzymes of vaccinia and rabbit pox. The second is the protein function which Kates & McAuslan (1967) postulated largely on the basis of the rapid shut down in DNA synthesis produced by puromycin at a time when stable and active enzymes are known to be present. Our findings with variola virus show that this second function is relatively insensitive to FPA. The results of Appleyard & Zwartouw (1965) taken in conjunction with those of Kates & McAuslan show that it must also be insensitive in the case of rabbit pox virus. As our own findings with
a strain of vaccinia suggest a similar pattern of behaviour it would appear that the relative insensitivity of this function to FPA may be of some general application to pox viruses. We were led to postulate the existence of a third protein function by the observation that the synthesis of DNA which proceeds in the presence of FPA was not accompanied by a parallel accumulation of ‘functional’ DNA. Admittedly this observation depended upon the sequential use of two different metabolic inhibitors, but we are encouraged in our belief that it is meaningful by the finding that pox viruses show apparently specific differences in the sensitivity of this function to FPA. The evidence presented in Fig. 2 (a) and (b) suggests that this function is normally expressed contemporaneously with the synthesis of DNA and may therefore be regarded as an

![Graph](image)

Fig. 2. The abilities of (a) variola, (b) vaccinia—Connaught Laboratories strain—and (c) rabbit pox—Utrecht strain to make functional DNA after the addition of FPA at various times early in the course of infection. ○—○, Functional DNA present at 8 hr following addition of FPA at the times plotted (24 hr titres after reversal of FPA inhibition and addition of FUDR or BUDR at 8 hr). ●—●, Functional DNA already present at the time of addition of FPA (24 hr titres following addition of FUDR or BUDR at the times plotted).

‘early’ function. In this analysis we have been careful to refer to ‘protein functions’ and not ‘proteins’. The second and third functions, both of which appear to require continued protein synthesis during the replication of virus DNA, may possibly be performed by a single protein.

The experiments reported in this note do not of course show which of the enzymes concerned in DNA synthesis are essential to the virus, nor do they help to elucidate the nature of either of the other two proposed protein functions. It is possible that a better characterization of the defective situations may provide some clue and this approach is being pursued. Meanwhile, our analysis of protein functions in the replication of virus DNA is relevant to studies of temperature-sensitive events affecting this process. Our results suggest three classes of defect which may be found in this situation and provide simple means of distinguishing between them. In the first two classes no virus DNA should be synthesized under restrictive conditions. An enzymic defect should be manifested by continued failure to synthesize DNA on return to permissive

On: Wed, 31 Oct 2018 05:41:40
conditions if FPA is added at the time of 'shift-down', whereas a defect in the second protein function should be shown by the synthesis of DNA in these circumstances. With the third type of defect, virus DNA should be synthesized under restrictive conditions but this DNA will appear non-functional if BUDR or FUDR is added and the cultures are returned to permissive conditions. It is of interest that in our studies of temperature-sensitive events with variola and alastrim viruses we have found situations which appear to correspond to the first and third classes of defect (Bedson & Cruickshank, in preparation; Cooper & Bedson, unpublished).

We thank Roche Products Ltd for a gift of 5-fluorodeoxyuridine, and Mr A. Dunn for his excellent technical assistance.

Department of Virology
The Medical School
University of Birmingham, England

REFERENCES

(Received 25 January 1968)