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AN ANALYSIS OF THE MPB-ARRESTED STAGE DURING REPLICATION OF VACCINIA VIRUS IN HeLa CELLS

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Summary: The stage of arrest caused by 2-mercapto-1-(β-4-pyridethyl) benzimidazole (MPB) treatment was analyzed during replication of vaccinia virus in HeLa cells. 1) The reversal from MPB-block of viral RNA polymerase (a late enzyme) induction was prevented by the addition of hydroxyurea (HU). 2) MPB did not influence the induction of viral DNA polymerase (an early enzyme) and the switch-off control for it, but co-addition of HU with MPB permitted the super-induction of the enzyme synthesis. These results suggest that the process of vaccinia virus DNA synthesis for replication in HeLa cells may be divided into at least two stages, an MPB-sensitive and an MPB-resistant stage.

Various metabolic inhibitors have been used for revealing the sequential molecular and biochemical processes which occur during the replication of vaccinia virus in cells (1,2). For this purpose, we (3) have used the anti-viral benzimidazole derivative, 2-mercapto-1-(β-4-pyridethyl)benzimidazole (MPB) (4-6). This compound reversibly disorganizes nucleolar structure by preventing ribosomal RNA synthesis (7) but does not significantly affect DNA and protein synthesis in HeLa cells (8). However, its mechanism of action has not yet been fully described.

The previous paper (3) demonstrates that MPB reversibly inhibits the synthesis of viral RNA polymerase and the production of infectious vaccinia virus in HeLa cells. The concentration range of MPB required for this inhibitory effect was the same as that needed for the disorganization of nucleoli.

In the present study, the stage of arrest caused by MPB treatment was analyzed during vaccinia virus replication in HeLa cells.

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MATERIALS AND METHODS

Monolayer cultures of HeLa cells were infected with vaccinia strain Lister at an MOI about 1 as previously described (3).

Treatment of HeLa cells with MPB has been described (3,5-7). Briefly, 75 µg MPB in 10 µl dimethylsulfoxide (DMSO) was added per ml of Eagle's minimum essential medium containing 2 mM glutamine and 2% calf serum (maintenance medium, MM) at zero time (the moment of infection) unless stated otherwise. For the control culture, DMSO was added. When the reversibility of the MPB-effect on cells was to be determined, the MPB-containing medium was removed and the cells were washed 3 times with MM containing 1% (v/v) DMSO and refed on MM.

For assaying viral RNA polymerase and DNA polymerase in cells, the cytoplasmic fraction was prepared as described by Jungwirth and Joklik (11). RNA polymerase activity as a late enzyme was assayed in cytoplasm by a procedure similar to that used by Pitkanen et al. (9). The cytoplasmic fraction was treated with 0.5% (v/v) Nonidet P-40 (NP-40) plus 0.5% (v/v) 2-mercaptoethanol (ME) for 10 min at 37°. The reaction mixture in a total volume of 500 µl contained 50 µmoles Tris-HCl, pH 8, 5 µmoles $MgCl_2$, 0.1% (v/v) NP-40, 0.1% (v/v) ME, 2 µmoles of ATP, GTP and UTP, and 5 µCi [^{3}H]CTP (17 Ci/mmol) and 100 to 150 µg protein (determined by the method of Lowry et al. (10)). The reaction mixture was incubated at 37° for 1 h after which 1.4 ml of 20% (v/v) cold perchloric acid (PCA) and 0.1 ml (100 µg) of bovine serum albumin were added. After 20 min at 0°, the precipitates were collected and washed 4 times with 3 ml of 5% (v/v) PCA. After solubilizing the precipitates with 0.5 ml of formic acid, radioactivity was determined by a scintillation counter.

DNA polymerase activity as an early enzyme was assayed in cytoplasm as described by Jungwirth and Joklik (11). After the reaction was carried out, acid insoluble materials were obtained and the radioactivity was determined as described above.

[^{3}H]CTP (17 Ci/mmol) and [^{3}H]TTP (30 Ci/mmol) were obtained from Radiochemical Center, Amersham, England, and nucleotides and HU were from Boehringer Mannheim. All reagents were of analytical grade. 2-Mercapto-1-(β-4-pyridethyl)benzimidazole was a generous gift from Prof. G. C. Mueller.

RESULTS AND DISCUSSION

Vaccinia virus-induced enzymes in infected cells are classified into two groups: early, which appear before the onset of viral DNA synthesis, and late, which require prior DNA synthesis (2,11,12). The previous paper (3) briefly describes the influence of MPB on the inductions of these early and late enzymes in HeLa cells.

To reveal the critical time for the inhibition of viral late enzyme induction, MPB was added to a series of cultures at various times after infection. The activity of viral RNA polymerase, a late enzyme (9), was determined at 9 h post infection in all cases. Figure 1 shows that the addition of MPB within 3 h after infection blocks the induction of enzyme. This effective time by MPB-treatment is quite similar to the results obtained in the paralleled experiment using hydroxyurea (HU).

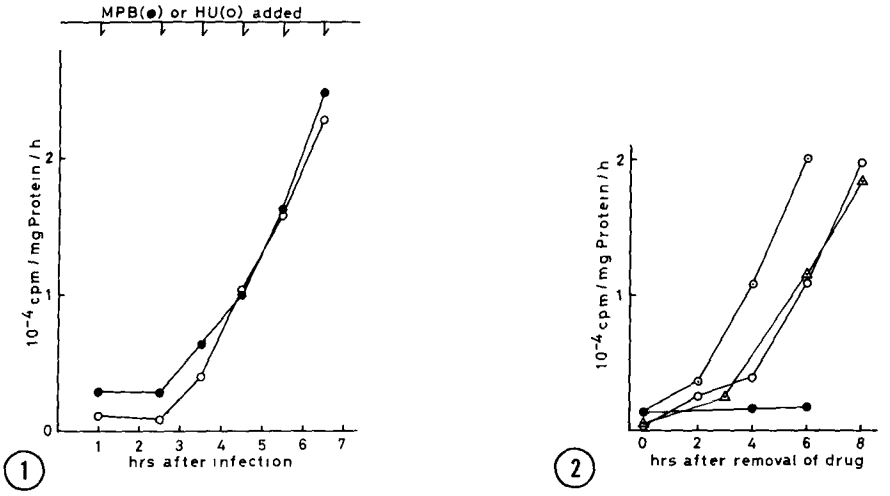

Fig. 1. Dependence of yields of RNA polymerase on the time of addition of MPB comparing HU. Vaccinia virus infected cultures were treated with either 75 μg/ml MPB (●) or 380 μg/ml HU (○) at the indicated time after infection. In each experiment, the assay of RNA polymerase activity was performed at 9 h post infection.

Fig. 2. Appearance of RNA polymerase after reversal of MPB or HU inhibition. HeLa cells were infected with vaccinia virus in the presence of MPB (75 μg/ml) or HU (380 μg/ml). At 9 h post infection, medium containing MPB (⊙) or HU (△) was replaced by drug-free medium and incubation was continued for the times indicated before measuring RNA polymerase activity. Additional cultures were treated with MPB at the time of infection and then with HU (380 μg/ml) at 8 h post infection. At 9 h post infection, the cells were washed and refed on medium containing HU (380 μg/ml) only (●). Control infected cultures contained no inhibitor (○). Zero h represents the time of infection.

It is known that HU reversibly prevents the development of vaccinia virus at an early process after infection by inhibiting viral DNA synthesis (13-15). The reversibility of MPB- or HU-inhibited cultures was compared by determining the appearance of viral RNA polymerase activity in cells after removal of the drug from the medium. Figure 2 shows that when HeLa cells were infected with vaccinia virus in the presence of MPB or HU for 9 h, washed with medium and then refed with fresh medium, viral RNA polymerase activity began to appear and linearly increased in both cases. However, the lag time required for the appearance of RNA polymerase after removal of MPB was shorter than that observed after removal of HU and that observed in control infected cultures.

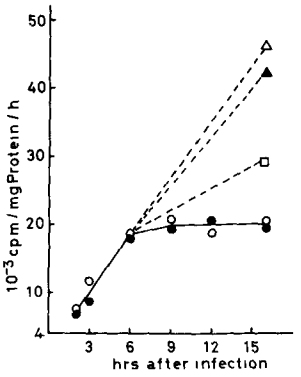

Fig. 3. Occurrence of DNA polymerase synthesis and "switch-off" control in the presence of MPB. HeLa cells were infected with vaccinia virus under various conditions. No drug (○); added MPB (75 μg/ml) at 0 time (●); added HU (380 μg/ml) at 0 time (△); added MPB and HU (▲) at 0 time; added MPB at 0 time and HU at 2 h post infection (□).

The effect of HU on the reversal from MPB-block was examined (Fig. 2). The results show that HU blocks the appearance of RNA polymerase activity after removal of MPB from inhibited cultures. This suggests that viral DNA synthesis directing RNA polymerase synthesis begins after removal of MPB from cultures. Thus, the sites of action of MPB and HU seemed to be quite similar in preventing viral DNA synthesis after infection in cells, however, the shortened lag time required for RNA polymerase synthesis in MPB-reversed culture suggest that some processes were advanced in infected cells during MPB-inhibition as compared to HU-inhibition.

It is well known that the synthesis of viral early enzymes ceases several hours after infection by a process that depends on viral DNA, RNA and protein synthesis (2,11). Therefore, if MPB acted as an inhibitor of DNA synthesis in a mechanism similar to that of HU in infected HeLa cells, the switch-off control for the early viral enzyme synthesis could not occur. As shown in Figure 3, there was no difference between the activity of viral DNA polymerase in vaccinia virus-infected cells treated with and without MPB. In other words, the switch-off control for preventing super induction of DNA polymerase operated in the presence of MPB as observed in control infection cultures.

However, with the simultaneous addition of HU to MPB-treated cultures, the switch-off for synthesis of DNA polymerase did not occur just as with cultures treated with HU alone. These results indicate that viral DNA synthesis that is related to the switch-off control occurred in the presence of MPB even though that required for late enzyme induction was blocked by the inhibitor.

Recent reports have demonstrated that vaccinia virus DNA is synthesized in nuclei as well as in cytoplasm early after infection (16-19), and that treatment of infected cells with the chemical inhibitor L-canaline can separate viral DNA synthesis into two classes (20), although their individual functions have not yet been defined. Our present observations suggest that viral DNA synthesis required for replication of vaccinia virus can be classified into MPB-resistant and MPB-sensitive stages, and that the MPB-sensitive stage may be affected by nucleolar function in host cells as described previously (3).

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