JOURNAL OF VIROLOGY, Jan. 1979, p. 405-408
0022-538X/79/0001-0405/04$02.00/0

Vol. 29, No. 1

Inhibition of Vaccinia Virus Growth by Zinc Ions: Effect on Early RNA and Thymidine Kinase Synthesis

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Received for publication 12 May 1978

Accumulation of thymidine kinase activity in vaccinia virus-infected cells was severely inhibited by zinc ions if the drug was added within 1 h postinfection. If added later, zinc ions had no effect on the enzyme synthesis. A fraction of RNA which is normally synthesized in infected cells, was missing from a proper part of the gradient if the cells were treated with zinc ions within 1 h postinfection (as has been shown by cosedimentation of pulse-labeled RNAs in isokinetic gradients). It is suggested that a transcriptional (or posttranscriptional) step is involved in zinc-caused inhibition of vaccinia virus growth.

Zinc ions inhibit growth of some RNA viruses (rhino-, picorna-, and arboviruses), where posttranslational cleavage of giant precursor polypeptides is prevented in the presence of the drug (1, 2). Zinc ions also inhibit growth of a DNA virus, Herpes simplex virus (HSV) (3, 9), where accumulation of large precursor proteins has not been found (3). A zinc concentration which inhibits HSV growth by 95% (0.1 mM) strongly inhibits synthesis of late proteins, especially P1-P4, but does not interfere with host protein synthesis (3). This leads to the suggestion that a process (or processes) other than protein synthesis itself, or posttranslational cleavage of precursor proteins, is affected by zinc ions in HSV-infected cells. It was also found that the effective concentration of the drug (0.1 mM) does not inhibit synthesis of an early protein, HSV-specified DNA polymerase (9). This finding creates a new situation since synthesis of some viral proteins (early) is permitted whereas synthesis of others (late) is restricted in the presence of zinc ions. The simplest explanation for the discrimination of late protein synthesis in the presence of zinc ions is the effect these ions have on viral DNA replication. The finding that synthesis of HSV DNA (identified by its buoyant density in CsCl) is not (or is just slightly) inhibited by this concentration of the drug (3, 9) does not eliminate such a possibility since synthesis of incompetent viral DNA with unchanged density cannot be excluded.

The aim of this study was to obtain some additional information concerning the mechanism of inhibitory action of zinc ions on multiplication of a DNA virus. Vaccinia virus has been chosen as a model since this virus induces cytoplasmic synthesis of nucleic acids, which makes it easier to distinguish between virus- and host-specified nucleic acids. Virus-induced thymidine kinase (TK) has been chosen as a representative of early viral proteins, since TK-deficient LM (TK^-) cells, a permissive host for the virus, lack both TK activity and inactive TK polypeptides (6). It is found here that zinc ions do not affect TK synthesis as such in vaccinia virus-infected cells, a finding similar to that with DNA polymerase in HSV-infected cells (9). However, the drug severely inhibits accumulation of TK if added within 1 h postinfection (p.i.). Moreover, if zinc ions are present during this early step of infection, a "light" RNA disappears from a proper part of the gradient.

Purified vaccinia virus (4) strain IHD clone 1 (7) and LM(TK^-) cells (5) were used in all the experiments. Propagation of cells and the virus, titration of the virus, TK assays, and viral RNA and DNA measurements were described earlier (10). Labeling of RNA for sedimentation analysis was performed in either infected (multiplicity of infection, 5 to 7) or uninfected cells. Cells were incubated for 1 h with or without zinc sulfate in minimal essential medium containing 5% calf serum. RNA was labeled with either [^{3}H]uridine (50 Ci/mmol, 2 μCi/ml, Amersham) or [^{14}C]uridine (50 mCi/mmol, 0.5 μCi/ml, Amersham) and added 1 h p.i. for 20 min. Under these conditions more than 90% of the cytoplasmic trichloroacetic acid-precipitable label probably was bound to membranes as shown by centrifugation of nucleus-free homogenates through 15 to 30% sucrose gradients (containing 10 mM Tris-hydrochloride, pH 7.6, 10 mM KCl, and 5 mM magnesium acetate) prepared on a 60% sucrose cushion (Superspeed 65, MSE, rotor 3 by 23, 4°C, 23,000 rpm, 2.5 h). Cytoplasmic

RNAs labeled by either ^{3}H or ^{14}C were isolated from nucleus-free homogenates by 1% sodium dodecyl sulfate, mixed, and analyzed in 15 to 30% RNase-free sucrose (Sigma) gradients containing 10 mM Tris-hydrochloride, pH 7.4, 0.1 M NaCl, 1 mM EDTA and 1% sodium dodecyl sulfate (Prepspin 50, MSE, rotor 6 by 38, 20°C, 23,000 rpm, 16 to 21 h). Gradients were fractionated into 1.3- or 1.1-ml fractions with monitoring at 254 nm, and radioactivity was precipitated, collected onto fiber glass filters, and counted in a toluene-based scintillator in a liquid scintillation spectrometer (Packard, Tricarb model 3380). Data presented in Fig. 1 are after appropriate corrections for spillover of ^{14}C counts into the ^{3}H channel (about 12%).

The minimal concentration of zinc sulfate which substantially inhibits vaccinia virus growth in infected cells was determined to be as low as 0.2 mM (Table 1). The chosen concentration (0.2 mM) stopped viral growth at any stage of the cycle but did not inactivate virus in vitro. This concentration was, however, toxic for cells after prolonged treatment ($\geq$10 h). Therefore, conservation of the ability of zinc-treated cells to support viral growth has been investigated by studying reversibility of the action of zinc ions. It was found that removal of the inhibitor within the first 5 h p.i. resulted in just slightly inhibited propagation of the virus, whereas later removal was ineffective (Table 2).

Table 3 shows that zinc ions, if added at the time of infection and 1 h p.i., strongly inhibited induction of TK in infected cells, whereas, if added 1.5 h p.i. and later, they had no effect on accumulation of the enzymatic activity. The inhibition of TK accumulation by zinc ions added within 1 h p.i. (lines 1 and 2) cannot be explained by possible interference with viral DNA replication since the latter is not required for TK synthesis (8, 10). Hence, a zinc-sensitive step that occurs earlier than viral DNA replication can be suggested. The results presented in Table 3 show also that zinc ions did not interfere with TK synthesis itself since more than 90% (line 3) and more than 95% (line 6) of TK were synthesized in the presence of the drug added 3 and 1.5 h p.i., respectively. This was interpreted to mean that zinc ions do not interfere with the translation process as such. An uninhibited accumulation of TK in the presence of zinc ions if the ions are added 1.5 h p.i. and later is similar to an

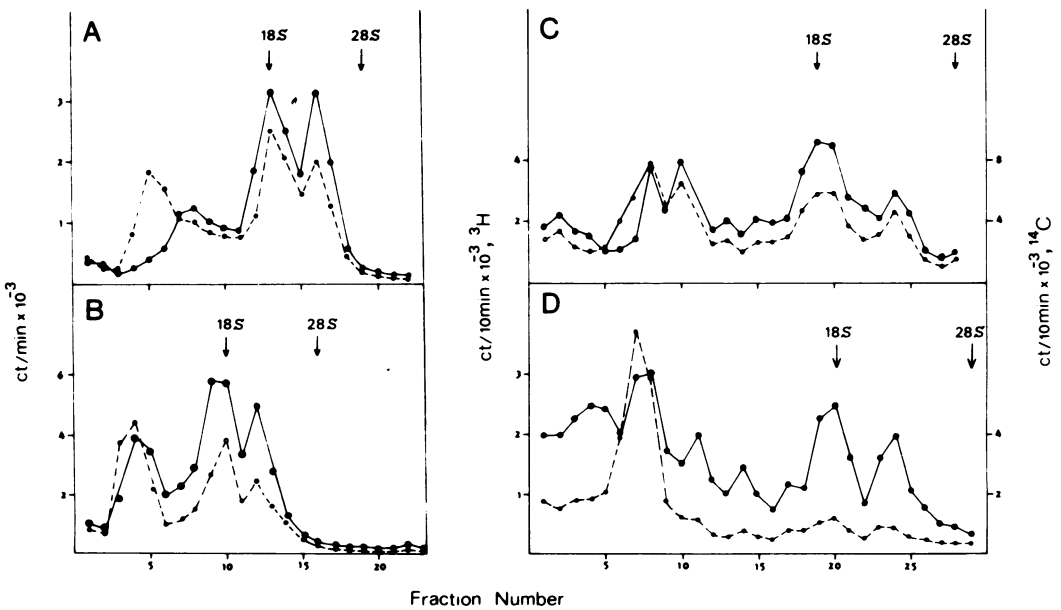

FIG. 1. *Effect of zinc ions on sedimentation of pulse-labeled RNAs from vaccinia virus-infected and noninfected cells. Cell monolayers (150-mm plates, Falcon) were infected at a multiplicity of infection of 5 to 7 and allowed to absorb virus from 1-ml volumes for 30 min. Unabsorbed virus was discarded, fresh maintenance medium (prewarmed) was added (zero time), and cells were incubated with or without zinc sulfate (final concentration, 0.2 mM) for 1 h. RNAs were labeled by either [^{3}H]- or [^{14}C]uridine for 20 min, extracted from nucleus-free homogenates, mixed, and analyzed in sucrose gradients (see text for details). (A) Infected, zinc-treated cells (^{3}H) and infected control (^{14}C); (B) infected, zinc-treated cells (both ^{3}H and ^{14}C); (C) infected untreated cells (both ^{3}H and ^{14}C); (D) noninfected, zinc-treated cells (^{3}H) and noninfected control (^{14}C). Symbols: ●, ^{3}H; ○, ^{14}C.*

TABLE 1. *Dose response of viral growth to zinc ions*[a]

$ZnSO_4$ (mM)	Viral yield at 24 h p.i. (PFU/ml)	
	Expt 1	Expt 2
0 (Infected control)	2.3×10^8	5.3×10^7
0.01	$>7.5 \times 10^7$	—[b]
0.05	$>7.5 \times 10^7$	—
0.1	3.5×10^7	7.0×10^6
0.15	—	1.0×10^6
0.2	7.0×10^5	7.0×10^5
0.25	4.5×10^5	7.0×10^5
0.3	—	5.0×10^5

[a] Cell monolayers (35-mm plates, Falcon) were infected at a multiplicity of infection of 5 to 7 and allowed to absorb virus from 0.05-ml volumes for 1 h. The unabsorbed virus was washed out by three successive washings, and minimal Eagle medium supplemented with 2% calf serum, antibiotics, and appropriate concentrations of $ZnSO_4$ was then added (zero time). The plates were incubated for 20 to 24 h (one cycle of growth for this virus is 13 to 15 h [10]), and the virus was titrated on L 929 cell monolayers. Residual virus, 5×10^5 PFU/ml (experiment 1) and 3.9×10^5 PFU/ml (experiment 2).

[b] —, Not tested.

TABLE 2. *Reversibility of inhibitory action of zinc sulfate on viral growth*[a]

$ZnSO_4$ (final concn 0.2 mM) at:		Viral yield (PFU/ml)	
Time of administration (h p.i.)	Removal (h p.i.)	Infected control at time of removal of the drug	Zinc-treated samples
0	2	—	3.3×10^7
—	—	6.0×10^5	—
0	5	—	1.4×10^7
—	—	5.5×10^5	—
0	10	—	7.5×10^5
—	—	3.8×10^6	—
0	24	—	7.5×10^4
—	—	7.8×10^7	—

[a] Infection was as it was described in footnote *a*, Table 1. Residual virus, 5.5×10^5 PFU/ml. Zinc sulfate was added at zero time to some plates while the remaining ones were left untreated (column 1). At times indicated in column 2 the drug was washed out, fresh maintenance medium was added, and plates were incubated overnight (column 4). Appropriate control plates (untreated) were frozen at times when the inhibitor was washed out from the treated samples (column 3).

uninhibited accumulation of HSV-induced DNA polymerase in the presence of zinc ions added 2 h p.i. (9). Unfortunately, it is not known whether earlier treatment of HSV-infected cells with zinc leads to an inhibition of DNA polymerase synthesis. The general suggestion from these data was that a step occurring earlier than protein synthesis is affected by zinc ions in vaccinia virus-infected cells.

It was shown that TK mRNA synthesis in WR-infected HeLa cells is completed within 2 h p.i. (8), and this is true also for IHD-infected LM (TK^-) cells (unpublished data). Since this very period is sensitive to an inhibitory action of zinc ions on TK accumulation, it was suggested that viral RNA synthesis might be involved. Direct measurements of total RNA synthesis showed, however, that zinc ions just moderately inhibit RNA synthesis in infected cells, which itself is an unlikely explanation for a prominent inhibition of TK production. Therefore, pulse-labeled cytoplasmic RNA from drug-treated infected cells was compared to that from nontreated infected cells (Fig. 1A). This panel shows that, in addition to unaffected distribution of 18S and 23S RNAs ("peak in peak"), distribution of "light" RNA is different in treated and untreated samples ("peak out of peak"). This is due to reduced ^3H radioactivity in fraction 5 (ratio of ^3H in fraction 5 to ^3H in fraction 13 or ^3H in fraction 16 is lower than the ratio of ^{14}C in fraction 5 to ^{14}C in fraction 13 or ^{14}C in fraction 16). It is not clear whether there is a shift of light RNA toward the bottom of the gradient (fractions 7 and 8) or if this RNA became visible due to reduction of ^3H radioactivity in fraction 5. Figure 1B shows distribution of both ^3H- and ^{14}C-labeled RNAs from zinc-treated infected cells, whereas distribution of both ^3H- and ^{14}C-labeled RNAs from nontreated infected cells is shown in Fig. 1C. Identical distributions were seen in both cases. It should be noted that

TABLE 3. *Effect of zinc ions on TK induction in vaccinia virus-infected cells*[a]

Expt no.	Time of administration of zinc (h p.i.)	TK activity (cpm/20 μl of extract		
		At time of administration of the drug	5.5 h p.i.	8 h p.i.
1	0	0	—	6,768
	1	0	—	7,848
	3	3,264	—	36,588
	5	16,386	—	35,346
	—	—	—	32,016
2	1.5	892	21,446	—
	—	—	23,561	—

[a] Infection was as it was described in footnote *a*, Table 1, except the washing-out step of unabsorbed virus was omitted. At times indicated (column 2) zinc sulfate was added to 0.2 mM to two plates, while one was used for TK measurements at the times of administration of the drug (column 3). Cells were incubated until either 8 (experiment 1) or 5.5 (experiment 2) h p.i., and the enzyme activities were measured in cytoplasmic extracts. Numbers in columns 4 and 5 are an average of two duplicate plates. The last line in each experiment shows untreated infected controls.

cytoplasm of noninfected cells contains measurable amounts of uridine incorporated into trichloroacetic acid-precipitable materials. Therefore, pulse-labeled cytoplasmic RNAs from zinc-treated noninfected cells (^{3}H) was compared to that from untreated noninfected cells (^{14}C) (Fig. 1D). Relatively low radioactivity (note the scales) makes a precise comparison rather difficult; however, the clear presence of both labels in fractions 7 and 8 allows us to say that light RNAs synthesized both in zinc-treated and untreated noninfected cells are indistinguishable by this method. The interpretation of the data presented in Fig. 1 is that synthesis of a fraction of RNA in zinc-treated infected cells is affected. This supports the idea of involvement of transcription in zinc-caused inhibition of vaccinia virus growth, although some posttranslational processes may alternatively be responsible for such inhibition.

I thank Y. Becker (Hadassah Medical School, Hebrew University, Jerusalem) for fruitful discussions and Alice Croitoru for technical assistance.

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