VACCINIA mRNA SYNTHESIS UNDER CONDITIONS WHICH PREVENT UNCOATING[1,2]

Bruce Woodson
Department of Microbiology, University of California Medical Center
San Francisco, California 94122

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The events by which the infecting DNA of poxviruses is rendered naked have been examined in detail (Joklik, 1964a, 1964b). Briefly, a 2-step "uncoating" mechanism is postulated; the viral "inducer protein", released during the first stage of uncoating, elicits the synthesis (by derepression of a portion of the host-cell genome) of a new protein, the "uncoating protein", which is instrumental in degrading virus cores. The second uncoating step renders the input viral DNA susceptible to DNase and is prevented by inhibition of protein synthesis, or when UV-inactivated infecting virus is employed, in which case the viral inducer protein is presumably damaged (Joklik, 1964c). The present experiments show, however, that even under these conditions, virus-specific mRNA is synthesized. It is concluded that viral DNA is capable of certain early functions while not yet fully uncoated.

METHODS

Strain S3 HeLa cells were cultivated in suspension (Eagle, 1959). The WR strain of vaccinia virus was grown in HeLa cells and purified in sucrose density gradients. The concentration of virus in purified stocks was determined by the O.D. at 260 mμ (Joklik, 1962). UV-inactivated virus was prepared from 5 ml aliquots of stock virus in sucrose by exposure to a G.E. germicidal lamp (max emission 2537 Å) for periods of 20 seconds, 40 seconds, or 80 seconds at a distance of 12 inches. Infectivity determined on primary chick embryo fibroblasts was reduced approximately $10^{2.5}$, $10^{3.5}$, $10^{4.5}$-fold respectively.

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[2]This work was reported at the FASEB meeting, April, 1967. See Fed. Proc. v.26, 1967.

For measurement of RNA or DNA synthesis, 5 ml aliquots of cells at 1 x 10^6/ml were used for pulse labeling for periods of 10 minutes with 0.5 µc of uridine-2-C^{14} or thymidine-2-C^{14} (specific activity 30 mc/mmole). Cytoplasmic extracts were prepared in 2 ml of RSB (Warner et al., 1963) or 10^{-3} M phosphate buffer pH 7.4 (Joklik and Becker, 1964) using a Dounce glass homogenizer. After centrifuging off nuclei and debris, radioactivity precipitable with TCA (5% final) was determined in 0.8 ml fractions (equivalent to 2 x 10^6 cells) by collection on Millipore filters and counting in a Beckman Low Beta II.

Carrier-free P^{32} was used for determination of base compositions. Cytoplasmic extracts prepared in RSB were centrifuged in 15-30% RSB sucrose density gradients at 4°C for 4 hours at 24,500 rpm. Gradients were analyzed with a Gilford recording spectrophotometer at 260 mµ. Polyribosome fractions were pooled, made 1% with SDS and 0.1 M with NaCl. RNA precipitated by 5-7 volumes of cold ethanol overnight was then hydrolyzed in KOH and analyzed according to Sebring and Salzman (1964).

RESULTS

Properties of the system. Vaccinia virus multiplies in the cytoplasm of cells and thus it is possible by pulse-labeling and preparation of cytoplasmic extracts to examine the rate and time of synthesis of virus-specific macromolecules (Becker and Joklik, 1964; Joklik and Becker, 1964). When viral DNA synthesis is blocked by FUDR (5-fluorodeoxyuridine) as in Fig. 1A, the observed RNA synthesis is that directed by parental DNA only. As the quantity of input DNA is increased, the rate and quantity of early mRNA transcribed from this DNA increases proportionately. Furthermore, regardless of the multiplicity, this early mRNA appears to be synthesized in a burst-like fashion, the bulk of it being made during the first hour after infection. Fig. 1B contrasts this early mRNA from parental genomes (curve a) with total mRNA synthesis, i.e., from both parental and progeny genomes (curve b). FUDR had no effect on uninfected cells (curve c).

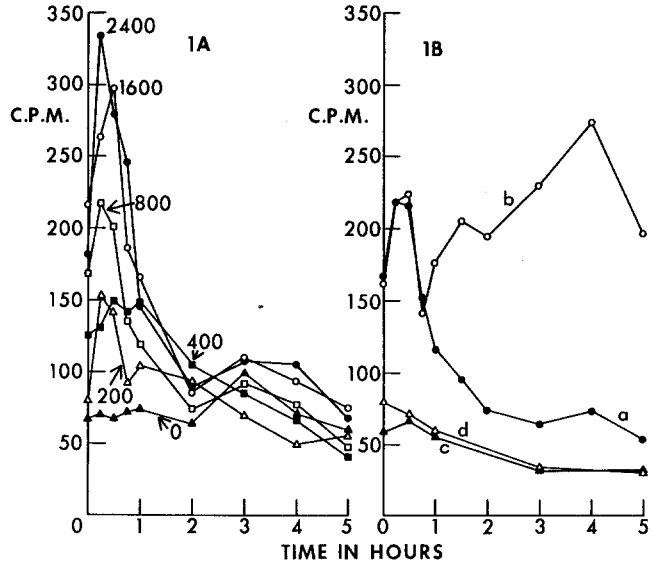

Fig. 1. The Effect of Multiplicity and FUDR on Vaccinia mRNA Synthesis. 1A). Six 6 ml cultures at 1 x 10^7 cells/ml were prepared in adsorption media (media supplemented with 1% serum and 0.02 M $MgCl_2$) containing 10^{-4} M FUDR. Virus at multiplicities of 0, 200, 400, 800, 1600, and 2400 particles/cell was allowed to adsorb for 15 min, at which time the cultures were diluted 10-fold in normal medium (zero time). At various intervals, aliquots were withdrawn and pulsed with C^{14} uridine. Cytoplasmic fractions were prepared and analyzed as described in the text. 1B). Four 6 ml cultures were prepared as in 1A. FUDR was added to a and c. Cultures a and b were infected at 1200 p/c, cultures c and d served as uninfected controls. Pulse-labeling, preparation and analysis of cytoplasmic fractions as in 1A.

UV-inactivated infecting virus. Fig. 2A shows the effect of UV-irradiation on the capacity of the virus to duplicate its DNA. Virus irradiated for 20 seconds (curve d) was able to synthesize a limited amount of DNA; however, with further irradiation (curves e and f) no DNA synthesis was detected. This result is not surprising in view of the report that UV-inactivated particles are not uncoated (Joklik, 1964c). However, Fig. 2B shows that UV-inactivated particles can function as a template in the transcription of early viral mRNA. Virus irradiated for 20 seconds (curve d) gave results similar to an unirradiated control with FUDR (curve c). Longer periods of irradiation, 40 seconds (curve e) and 80 seconds (curve f), led to a prolonged synthesis of RNA. Similar results were obtained in the presence of FUDR.

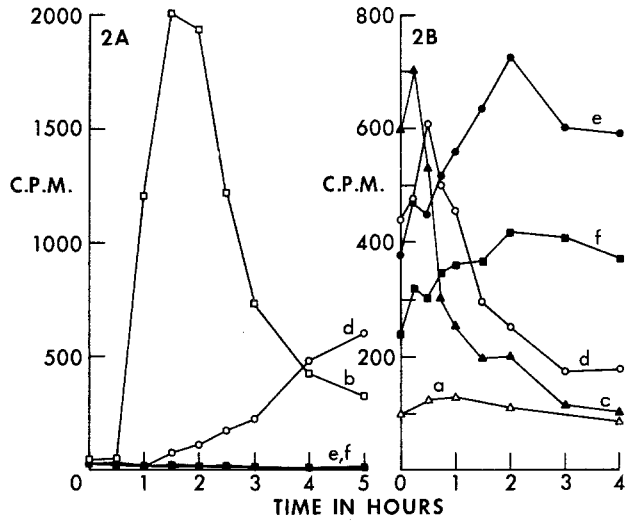

Fig. 2. The Effect of UV-irradiation on Vaccinia DNA and mRNA Synthesis. Six cultures were prepared in adsorption media as in Fig. 1; 3 with 6 ml, 3 with 10 ml. The 3 (6 ml) cultures received (a) 10^{-4} M FUDR, (b) vaccinia at 1200 p/c, (c) vaccinia 1200 p/c plus 10^{-4} M FUDR. The 10 ml cultures received (d) 1200 p/c vaccinia UV-irradiated 20 sec, (e) 1200 p/c irradiated 40 sec, (f) 1200 p/c irradiated 80 sec. After 15 min adsorption, all cultures were diluted 10-fold in normal medium and at various intervals aliquots from a, c, d, e, and f were pulse-labeled with C^{14} uridine (Fig. 2B); b, d, e, and f with C^{14} thymidine (Fig. 2A). Cytoplasmic fractions were prepared and analyzed as described in the text.

The effect of puromycin and actidione. Fig. 3A shows that inhibition of protein synthesis by either puromycin or actidione (cycloheximide) completely abolishes the capacity to synthesize viral DNA (curves d and e). This is in keeping with Joklik's suggestion (Joklik, 1964 a,b) that under such conditions the "uncoating protein" is not synthesized and thus viral cores are not degraded. Fig. 3B shows, however, that even under these conditions viral mRNA is transcribed from the input DNA. Curve c is the control; d is with puromycin; e with actidione. Protein synthesis in both d and e was inhibited by 96%.

Evidence that the RNA synthesized in the presence of puromycin or actidione, or with UV-inactivated infecting virus is vaccinia mRNA. Table I shows the base composition of polysome-associated RNA under the variety of

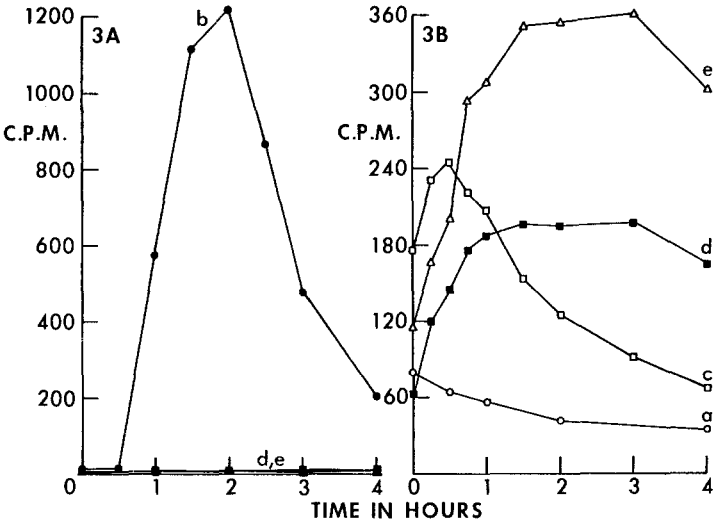

Fig. 3. The Effect of Actidione and Puromycin on Vaccinia DNA and mRNA Synthesis. Five cultures prepared as in Fig. 1 (a, b, and c with 6 ml, d and e with 10 ml) received (a) and (c) 10^{-4} M FUDR, (b) nothing, (d) 500 µg/ml puromycin, (e) 1000 µg/ml actidione. After 10 min exposure to the inhibitors, separate aliquots from b, d, and e were pulse-labeled with C^{14} amino acids for 10 min. Protein synthesis in d and e was inhibited by 96%. Virus at M = 1200 p/c was added to b, c, d, and e and allowed to adsorb for 15 min. All cultures were then diluted 10-fold with normal medium and at various intervals aliquots of a, c, d, and e were pulse-labeled with C^{14} uridine (Fig. 3B); aliquots of b, d, and e with C^{14} thymidine (Fig. 3A). Cytoplasmic fractions were prepared and analyzed as described in the text.

conditions discussed above. In all cases the base composition, clearly different from that of uninfected cells, reflects the composition of vaccinia DNA.

DISCUSSION AND SUMMARY

There can be no doubt that RNA synthesis, presumably viral mRNA from parental DNA, proceeds under the conditions which are reported to inhibit uncoating. In fact, these studies reveal several interesting aspects of early mRNA synthesis. First, the experiments with puromycin and actidione, in which protein synthesis was inhibited by 96% before the addition of virus, indicate that this synthesis is catalyzed by pre-existing enzyme(s). A second aspect relates to control, which is apparently lost when protein synthesis is inhibited or when UV-inactivated virus is employed. This was true

	CULTURE CONDITIONS	Moles/100 moles nucleotides A	U(T)	G	C	A+U / G+C
Exp. 1	a. Uninfected control	25.1	22.7	27.0	25.3	0.92
	b. Infected control	35.6	27.2	19.2	18.1	1.69
	c. Infected + FUDR	34.9	23.3	20.1	21.7	1.39
	d. UV-inactivated virus	42.2	21.9	18.3	17.6	1.79
	e. Infected + puromycin	36.5	23.9	19.4	20.2	1.53
Exp. 2	Uninfected control	26.4	22.4	24.7	26.3	0.96
	Infected + puromycin	36.5	26.6	19.7	19.7	1.54
	Infected + actidione	35.5	25.8	19.0	19.7	1.58
	*VACCINIA DNA	31.8	31.0	18.5	18.7	1.69

Table I. Base Composition of Polysome-associated RNA.
Exp. 1) Five 10 ml cultures (as in Fig. 1) were prepared in PO_4-free adsorption media. Culture c received 10^{-4} M FUDR; e, puromycin at 500 μg/ml. After 10 min exposure to the inhibitors, vaccinia at 1200 p/c was added to b, c, and e; vaccinia UV-irradiated 80 sec was added to d. After a 15 min adsorption period, all cultures were diluted 10-fold with PO_4-free medium (zero time). Cultures c, d, and e received 1 mc P^{32} for 2 hrs beginning 15 min after zero time; cultures a and b for 2 hrs beginning 2 hrs after zero time. Polyribosomes were prepared from cytoplasmic fractions by centrifugation on sucrose gradients. The base composition of the polysome-associated RNA was determined as described in the text.
Exp. 2) Three 10 ml cultures were prepared in PO_4-free adsorption media. One culture received puromycin (500 μg/ml), another received actidione (1000 μg/ml). After 10 min all three cultures received virus at M = 1200 p/c. Adsorption and dilution as in Exp. 1. One mc P^{32} was added to each culture for 2 hrs beginning 15 min after zero time dilution.

*Joklik, W.K. (1962) Virology 18, 9.

whether FUDR was present or absent.

Finally, it should be noted that the concept of the involvement of the host genome in the uncoating process rests on the assumption that DNase susceptibility is a precondition for the functional activity of the input DNA. The present evidence shows, on the other hand, that a normal or greater than normal quantity of early mRNA is synthesized under conditions which have been reported to prevent conversion to a DNase susceptible state. Preliminary experiments indicate that this mRNA synthesis is not due to anomalous uncoating of the input virus by a "derepressed" host cell (Joklik,

1964 a,b). In view of these results, it would seem appropriate to re-examine the concept of uncoating and the evidence involving the host genome in this mechanism.

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