Virus-Associated Nucleases: Evidence for Endonuclease and Exonuclease Activity in Rabbitpox and Vaccinia Viruses

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The endonucleolytic action of a deoxyribonuclease activity in rabbitpox and vaccinia virus was established by change in sedimentation rate of denatured ^3H -lambda deoxyribonucleic acid substrate. The presence of two deoxyribonuclease activities in pox-virus is confirmed. Exo- and endonuclease activities are unmasked by treatment of purified virus with the detergent Nonidet P-40 and further enhanced by treatment of viral "cores" with trypsin.

Several unrelated viruses have been reported to have nuclease activity intimately associated with their virions. For example, adenovirus, porvirus, and Rous sarcoma virus have deoxyribonuclease activity (2, 9, 10). Three nucleases are elicited by poxvirus infection (6). Of these the pH 5 exonuclease, a "late" activity that has been purified extensively and characterized (8), and the pH 7.5 endonuclease are thought to be intimately associated with the viral core (10).

Before one can speculate on the function of these nucleases in virus replication, it is necessary to establish, unequivocally, the nature of the action of these enzymes and their location in the virion. The argument advanced (10) in support of a vaccinia virus-associated endonuclease rests on two points. First, there is an initial lag shown in the kinetics of liberation of acid-soluble fragments from substrate deoxyribonucleic acid (DNA) by viral enzyme at neutral pH values. We have confirmed this but suggest that, although such a result is consistent with an endonucleolytic reaction, other interpretations are possible. Second, there appears to be a relatively rapid drop in molecular size of substrate as determined by the membrane filtration technique. We suggest that data obtained by this technique (3, 10) may be misleading since Nonidet P-40 (NP-40), used to activate the viral enzyme, by itself causes a marked loss in retention of DNA by nitrocellulose filters. That is to say it gives a false indication of reduction in the size of substrate DNA.

Preliminary studies on the response of viral nuclease activity to pH gave results essentially similar to those of Pogo and Dales (10), although we found that, under conditions in which initial reaction rates are determined, the ratio of activity

at pH 5 to activity at pH 7.5, as measured by formation of acid-soluble products (8), was between 5 and 10:1. In contrast, Pogo and Dales (10) found a comparatively high rate of conversion of DNA to acid-soluble products in the range pH 7 to 8, indicative of either exonuclease activity or high endonuclease activity.

We tested for endonuclease activity by (i) the technique of Birnboim (1) with gel filtration and (ii) the reduction in molecular weight of substrate DNA by velocity sedimentation under conditions in which the formation of acid-soluble products was of the order of only 10% or less.

The Utrecht strain of rabbitpox (RP) and the WR strain of vaccinia virus (WR) were grown in hamster kidney monolayer cultures or HeLa-S₃ suspension cultures, respectively. Virus was subjected to two cycles of purification by the procedures of Katz and Moss (5). Such preparations had a titer of approximately 5×10^8 pock-forming units per ml and a protein concentration of 0.8 mg/ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.

Detergent-activated virus (identical results were obtained with either RP or WR) was incubated with ^3H -DNA, and the reaction mixture (Fig. 1) was applied to Sephadex G-100. A gradual reduction in rate of elution of the products with time of incubation was evident (Fig. 1) and consistent with that expected from endonucleolytic action; practically no reaction product corresponding to mononucleotides was formed even on prolonged incubation. On the other hand, reaction products produced at pH 5 eluted from the gel at the position expected for mononucleotides (results not shown), consistent with an exonucleolytic action.

As an alternative approach, detergent-activated

FIG. 1. Gel-filtration of reaction products. Poxvirus suspensions were adjusted to 0.5% NP-40 and 0.02 M mercaptoethanol. The mixture was incubated at 37°C for 30 min. Virus (20 µg of viral protein) thus activated was incubated with 0.02 M mercaptoethanol, 0.5% NP-40, 0.16 M Tris-hydrochloride buffer (pH 7.5), and ^3H -lambda DNA (5 µg, 30,000 counts/min denatured by adjustment to 0.05 M NaOH for 10 min at room temperature and then readjusted to pH 7.5 with Tris-hydrochloride buffer). This mixture (total volume 130 µliters, final pH 7.5) was incubated for the times indicated at 37°C, adjusted to 2.5% sodium dodecyl sulfate, and then applied to Sephadex G-100 columns (15 × 0.5 cm) for elution as described by Birnboim (1). Fractions (approximately 0.3 ml) were collected for determination of radioactivity. Isotopic adenosine triphosphate was used as a marker.

RP was incubated with denatured ^3H -lambda DNA at several concentrations of virus, and the products of the reaction were sedimented through alkaline sucrose gradients. Under conditions causing little liberation of acid-soluble fragments, there was a marked reduction in the molecular weight of the substrate (Fig. 2). Further, there was a good correlation between reduction in sedimentation rate of substrate and virus concentration as shown and with incubation times between 0 and 60 min. The reaction was absolutely dependent on activation by detergent. Native DNA was not a substrate for the endonuclease, since no reduction in molecular size was observed with either neutral gradients to detect double-strand breaks or alkaline gradients to detect single-strand breaks in double-strand molecules.

The concentration of detergent used is known to liberate the VSP6 group of poxvirus structural polypeptides (4; W. K. Joklik and B. Moss, *personal communications*). Liberation of nuclease from poxvirus was attempted with either NP-40 treatment alone or in conjunction with trypsin digestion. The results (Table 1) indicate that some exonuclease activity (measured at pH 5) was released by such treatments. However, activities at either pH 5 or 7.5 (the optimum values for exonuclease and endonuclease, respectively) were stim-

ulated 10-fold (experiments 3, 6, 7) by trypsin treatment of subvirions. It is possible that trypsin treatment not only enhances core activity by destroying an inhibitor but also destroys any liberated nuclease.

These data lend strong support for the endonucleolytic action of a latent enzyme activity in poxvirus particles. The activation of latent exonuclease and endonuclease activities by removal of certain coat polypeptides with detergent and the retention of nuclease by cores after partial trypsin digestion constitute strong evidence in favor of the incorporation of these activities within the virions. In confirmation of results published (10), we found stimulation of enzyme activities after trypsin treatment of detergent cores. Nuclease activity at pH 7.5 (endonucleolytic) was stimulated 10-fold when assayed by liberation of acid-soluble fragments; we have not rigorously excluded the possibility that this might represent the activation of yet another nuclease as well as the original endonuclease

FIG. 2. Velocity sedimentation of reaction products. Rabbitpox was activated with detergent and incubated with denatured ^3H -lambda DNA as described in the legend to Fig. 1. The incubation time was 30 min, and, after the sodium dodecyl sulfate step (see Fig. 1), the reaction mixtures were layered onto linear sucrose gradients [9 ml, 5 to 20% (w/v) sucrose in 0.9 M NaCl, 0.001 M ethylenediaminetetraacetic acid, and 0.1 M NaOH], and centrifuged (SW41 rotor Spinco L-2, 40,000 rev/min, 150 min). Δ , 10 µg; $\circ$, 20 µg; $\times$, 40 µg of viral protein; $\bullet$, virus omitted. Fractions were collected from the bottom of the gradient, and the radioactivity was determined by liquid scintillation spectrometry. Under the conditions of assay, no acid-soluble fragments were formed.

TABLE 1. *Liberation of nuclease from poxvirus by NP-40 treatment alone or in conjunction with trypsin digestion*^a

Treatment	Acid-soluble counts/min	
	pH 5	pH 7.6
NP-40 supernatant fluid	3,300	150
NP-40 interface.....	4,500	154
NP-40 pellet.....	10,600	3,600
Trypsin supernatant fluid..	234	150
Trypsin interface	314	140
Trypsin pellet.....	23,400	24,100
Virus plus NP-40	9,900	2,010
Virus, no treatment.....	0	0

^a Rabbitpox was activated with detergent and mercaptoethanol (legend to Fig. 1), diluted four-fold, layered over a 1-ml cushion of 30% (w/v) sucrose, and centrifuged (SW65 rotor, 60,000 rev/min, 60 min, 10°C). Three milliliters from the top of the gradient (supernatant fluid NP-40), 1.0 ml including the sucrose interface (NP-40 interface) and the pellet from the bottom of tube (pellet NP-40) were collected. The pellet was resuspended in water (1.0 ml), and 0.5 ml was treated with trypsin (50 µg in 0.5 ml of 0.02 M Tris-hydrochloride, pH 8, 15 min, 37°C). This was then diluted to 3.0 ml and centrifuged over a sucrose cushion as above. Fractions were taken from the top 3.0 ml (trypsin supernatant fluid) or the interface (trypsin interface). The pellet was resuspended in 0.5 ml of 0.01 M Tris-hydrochloride, pH 7.5 (trypsin pellet). Samples of these fractions were assayed for nuclease activity. The volume of enzyme taken for each assay was such that each represented the same amount as would have been taken from the original stock of virus. The reaction mixture described under Fig. 1 was run either at pH 5 (in 0.01 M sodium acetate buffer) or at pH 7.5, and the incubation time was 60 min. At the end of the reaction, the mixtures were chilled, and acid-soluble counts were determined as described previously (8).

but assume that the activity we measured represents enhanced endonuclease.

Both poxvirus and frog virus 3 have at least one virion-associated enzyme in common (7). On the assumption that viral nucleases have an essential function for replication or recombination, it would be of interest to compare these two unrelated cytoplasmic deoxyviruses (poxvirus and frog virus 3) to determine whether they possess similar nuclease activities.

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