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Soluble Endoribonuclease Activity from Vaccinia Virus: Specific Cleavage of Virion-Associated High-Molecular-Weight RNA

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A soluble endoribonuclease activity was extracted from purified vaccinia virus cores by treatment with sodium-deoxycholate and dithiothreitol. The soluble enzyme readily cleaved purified virion-associated high-molecular-weight RNA to limit-sized fragments sedimenting at 8 to 12S. Purified virion-released 8 to 12S polyadenylated mRNA was not degraded by the enzyme extract. The soluble endoribonuclease did not require the presence of ribonucleoside triphosphates for activity.

In vitro, purified vaccinia virus synthesizes RNA, the bulk of which sediments at 8 to 12S. Under appropriate pulse-label conditions, however, high-molecular-weight RNA is labeled preferentially (4). Transcription in vitro of either monocistronic or polycistronic RNAs has been shown to be regulated by the concentration of ATP (manuscript in preparation). The high-molecular-weight RNA is virion associated and is not released from the virus as high-molecular-weight RNA. It can, however, be chased into 8 to 12S RNA fragments, which are then released from the virus. This cleavage of high-molecular-weight RNA by the virus was shown to require ribonucleoside triphosphates and could be inhibited by ethidium bromide (4, 5). The ability to chase virion-associated high-molecular-weight RNA to virion-released monocistronic-sized RNA fragments and the homology of the transcribed sequences represented in the two size classes of RNA (6) suggested a precursor-product relationship. Implicit in this schema was the existence of a specific virion-associated endoribonuclease activity capable of excising monocistronic mRNA fragments from a polycistronic precursor. In this communication, we have demonstrated the existence of such a specific endoribonuclease activity associated with purified vaccinia virus.

Soluble endoribonuclease activity was extracted from purified vaccinia virus cores by disruption with sodium-deoxycholate and dithiothreitol as described (7). The soluble enzyme fraction was passed through a DEAE-cellulose column to remove endogenous DNA, and the flowthrough containing the enzyme was used directly. Virion-associated high-molecular-weight RNA was synthesized in vitro, extracted with phenol, and precipitated twice from 2 M LiCl (1) and once as the Na salt by ethanol precipitation. Virion-released 8 to 12S polyadenylated [poly(A)$^+$] mRNA was phenol extracted from a virus-free supernatant from an in vitro RNA polymerase reaction and purified by two successive sedimentations on 15 to 30% sucrose gradients. The poly(A)-containing fraction from this RNA was selected on poly(U)-Sepharose columns.

The sedimentation profile of purified virion-associated high-molecular-weight RNA obtained on sodium dodecyl sulfate (SDS)-sucrose gradients is shown in Fig. 1. From its sedimentation rate and methylated cap structure analysis (3), these RNA molecules contain approximately 4,000 bases. When this RNA was incubated in the standard endoribonuclease reaction but without enzyme extract, no appreciable alterations in sedimentation profile were observed (Fig. 1). Incubation in the presence of soluble enzyme extract obtained from vaccinia virus cores resulted in a rapid cleavage of the high-molecular-weight RNA to RNA fragments of approximately 1,000 bases sedimenting as 8 to 12S molecules. More extended incubation periods under these conditions or addition of fresh enzyme did not result in additional cleavage of the RNA beyond 8 to 12S (Fig. 1). The data presented in Fig. 1 suggest that no appreciable amounts of low-molecular-weight RNA fragments are generated by the endoribonuclease activity and, furthermore, that there appears to be a limit-size fragment of approximately 1,000 bases which is generated by endonucleolytic cleavage.

Additional evidence for the specificity of the vaccinia endoribonuclease activity is demon-

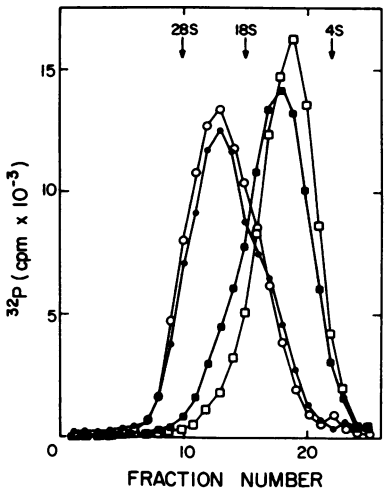

FIG. 1. *Endoribonucleolytic cleavage of purified virion-associated high-molecular-weight RNA. High-molecular-weight virion-associated RNA was labeled with [α-^{32}P]UTP and purified from pelleted virus as described in the text. The reaction for soluble endoribonuclease activity consisted of 50 mM Tris-hydrochloride (pH 8.4), 10 mM Mg^{2+}, 10 mM dithiothreitol, 5 mM GTP, 5 mM ATP, [^{32}P]UTP-labeled purified high-molecular-weight RNA, and 10 μl of the soluble vaccinia enzyme extract from the DEAE-cellulose flowthrough fraction. The reaction (100 μl) was incubated at 37°C and inactivated by the addition of 4 volumes of 0.1% SDS and 10 mM Na_3EDTA. The samples were boiled for 20 s, followed by rapid quenching in ice water, and analyzed on 15 to 30% SDS-sucrose gradients at 20,000 rpm for 18 h at 25°C in a Spinco SW40 rotor. Fractions were collected from the bottom and counted in 10 ml of Aquasol with 1 ml of water. The figure shows the sedimentation profiles of purified virion-associated RNA obtained when SDS and EDTA were present (●) prior to addition of enzyme, (○) after incubation at 37°C for 30 min in the absence of enzyme, and after (■) 30 min and (□) 60 min of incubation in the presence of enzyme. Sedimentation profiles of radiolabeled HeLa ribosomal RNA markers were obtained on parallel gradients.*

strated in Fig. 2. Whereas soluble enzyme extracts from vaccinia virus cores readily cleaved high-molecular-weight RNA, no cleavage of purified virion-released 8 to 12S poly(A)$^+$ mRNA could be demonstrated. This suggests that the endoribonuclease activity possesses a strict substrate requirement in terms of nucleotide sequence, conformation, or size.

Previously published data suggested that virion-associated high-molecular-weight RNA could be chased to virion-released 8–12S-size fragments. The ability to show such cleavage of the high-molecular-weight RNA by virus recovered from an in vitro RNA polymerase reaction was shown to be dependent on the presence of ribonucleoside triphosphates (4). It was not clear, however, whether the ribonucleoside triphosphate requirement was an immediate cofactor for the endoribonuclease activity or whether it was indirectly related. This was difficult to assess, since the virus contains a large number of ribonucleoside triphosphate-utilizing functions. The availability of a soluble endoribonuclease activity allowed us to determine whether ribonucleoside triphosphates were intimately associated with endoribonuclease activity. Purified high-molecular-weight RNA was incubated with soluble enzyme extracts either in the absence or the presence of ATP and GTP. Endoribonucleolytic cleavage of the high-molecular-weight RNA was evident either in the absence or in the presence of ribonucleoside triphosphates (Fig. 3). These data suggest that the requirement for ribonucleoside triphosphates for cleavage of the high-molecular-weight RNA by virus recovered from an in vitro RNA polymerase reaction as previously described (4) is not due to a direct requirement for the endoribonuclease activity itself and probably represents some other rate-limiting ribonucleoside triphosphate-dependent step associated with the processing mechanism. One possible step might be the RNA extrusion system described by Kates and Beeson (2).

The nature of the endoribonuclease activity in terms of its genetic origin, chemical specificity, and biological function can only be answered by future additional experiments. Preliminary studies on the nature of the RNA substrates suscep-

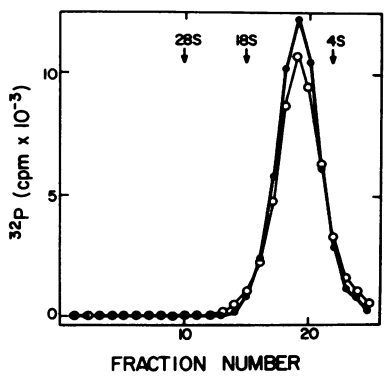

FIG. 2. *Lack of endoribonucleolytic activity with purified virion-released 8 to 12S poly(A)$^+$ mRNA. Virion-released 8 to 12S poly(A)$^+$ mRNA labeled with [α-^{32}P]UTP was purified by two successive fractionations on 15 to 30% SDS-sucrose gradients, followed by selection of poly(A)$^+$ species by absorption and elution from poly(U)-Sepharose. The sedimentation profiles of this RNA incubated at 37°C for 30 min in the (●) absence or (○) presence of 20 μl of enzyme extract are shown.*

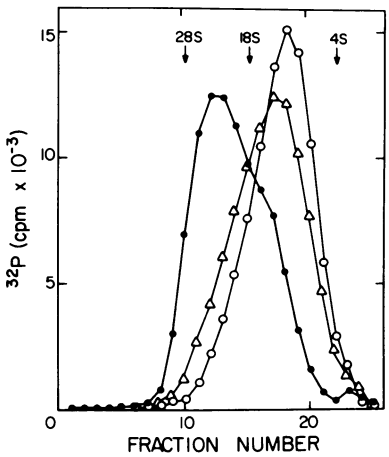

FIG. 3. *Endoribonucleolytic cleavage of purified virion-associated high-molecular-weight RNA in the absence of ribonucleoside triphosphates. High-molecular-weight RNA prepared as in the legend to Fig. 1 was made 0.1% with SDS and 10 mM with Na_3EDTA* (●) *immediately or after incubation at 37°C either* (△) *with or* (○) *without 5 mM ATP and 5 mM GTP.*

tible to the endoribonuclease activity reported here have indicated no endo- or exoribonuclease activity against any of the available single- or double-stranded synthetic polynucleotides tested, nor was any activity detected with 4, 18, or 28S ribosomal RNA from HeLa cells. Endoribonuclease activity was detected, however, using hn RNA (>28S) purified from late adenovirus-infected HeLa cells. The resultant limit-cleavage products were approximately 20 and 14 to 16S as determined by SDS-sucrose gradient sedimentation (M. Sarma and N. K. Chatterjee, unpublished data). These observations provide additional support for the specificity of the endoribonuclease reported in this communication. The purification of this endoribonuclease activity is currently in progress.

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