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Terminal Riboadenylate Transferase: a Poly A Polymerase in Purified Vaccinia Virus

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Purified vaccinia virus treated with Triton X-100 catalyzes the incorporation of ATP into an acid-insoluble product. The enzymatic activity responsible for the ATP polymerization is demonstrated to be different from vaccinia RNA polymerase in its preferential use of ATP as substrate and on the basis of heat stability, pH optima, and metal ion requirement. The ATP polymerization reaction is stimulated 10-fold by the addition of $rA(pA)_5$. In accordance with our earlier terminology, we call this Mn^{2+}-dependent enzyme terminal riboadenylate transferase to distinguish it from Mg^{2+}-dependent poly A polymerase.

The presence of RNA polymerase activity and the synthesis of free and attached poly A sequences by vaccinia virus cores has been described by Kates and co-workers (10, 11). The poly A sequence on vaccinia mRNA has been shown to be at the 3'-terminus (18). This sequence could then be synthesized by the viral RNA polymerase by transcribing dT sequences in vaccinia DNA or be added posttranscriptionally by a separate enzyme (10).

Two cellular enzymes have been described which polymerize ATP in the absence of a DNA template, one depending on Mg^{2+} for activity and the other on Mn^{2+} (6). The Mn^{2+}-dependent enzyme from calf thymus gland has been purified to homogeneity in this laboratory and named terminal riboadenylate transferase (TrT) to distinguish it from the Mg^{2+}-dependent poly A polymerase (20).

The work presented here demonstrates that there is a poly A polymerase activity, separate from RNA polymerase, present in vaccinia virus cores. Because the enzyme is Mn^{2+} dependent, we classify it as a terminal riboadenylate transferase as a manner of convenience in communication and not to imply a definite relationship to the calf thymus TrT.

MATERIALS AND METHODS

Virus and cells. Vaccinia virus (strain WR) was grown in Earle L cells (clone 929) (19) in suspension culture and titrated by a plaque assay on LLC-MK_2 cell (8) monolayers. Both cell lines were grown in Swim 67 medium (17) supplemented with 7.5% calf serum. For virus infection, L cells were collected by centrifugation and suspended at 5×10^7/ml in growth medium containing 10 PFU of vaccinia virus per cell. Virus adsorption was allowed to proceed for 20 min at 37 C and then the cells were diluted to 10^6/ml. The infected cells were harvested 24 h later by centrifugation, washed with 0.25 M sucrose in 25 mM KCl, 5 mM $MgCl_2$, and 50 mM Tris-hydrochloride (pH 7.5), resuspended in the same medium, and disrupted with a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at $600 \times g$ to remove nuclei, and vaccinia virus was purified from the cytoplasmic fraction by sucrose gradient centrifugation as described by Joklik (9). Virus from the major opalescent peak was sedimented at $30,000 \times g$ for 15 min and stored in 0.05 M Tris-hydrochloride (pH 8.6) at −20 C.

Preparation of virus for enzyme assay. The purified vaccinia virus was converted to cores by treatment with 1% Triton X-100 and 10 mM mercaptoethanol for 30 min at 35 C. The virus cores were then sedimented at $30,000 \times g$ for 15 min and resuspended in 0.05 M Tris-hydrochloride, pH 8.6, or assayed directly for enzymatic activity. This treatment resulted in a 50-fold increase in activity in the TrT assay over that demonstrated by the intact virion. RNA polymerase could not be demonstrated in the untreated virus. One optical density unit (ODU) of vaccinia virus is the product of the optical density of the suspension at 260 nm before treatment with Triton and the volume expressed in milliliters (15).

Substrates. Radioactive nucleoside triphosphates were obtained from Schwarz/Mann and dried down to remove ethyl alcohol before use. $rA(pA)_5$ was purchased from Miles Laboratories and $rA(pA)_{12}$ was prepared as previously described (2).

Enzyme assays. The RNA polymerase reaction mixtures contained 0.1 mM ^{3}H-UTP (179 counts per min per pmol); 0.4 mM GTP; 0.4 mM CTP; 0.8 mM ATP; 4 mM $MgCl_2$; 7 mM mercaptoethanol; 0.1 M Tris-hydrochloride, pH 8.8; 10 mM phosphoenolpyruvate; 25 μg of pyruvate kinase (Miles Laboratories); and 0.05 ODU of vaccinia virus cores in a total of 250 μliters. Endogenous viral DNA was the template for this reaction.

Unless noted otherwise, the TrT reaction mixtures contained 0.5 mM ^{3}H-ATP (25 counts per min per pmol); 0.01 mM $rA(pA)_5$ or $rA(pA)_{12}$; 0.5 mM $MnCl_2$; 0.2 M Tris-hydrochloride, pH 8.0; and 0.05 ODU of vaccinia virus cores in a total volume of 250 μliters. Reaction progress was followed by taking samples at various times onto GF/C filters and processing for acid-insoluble product (3).

RESULTS

RNA polymerase and TrT activity in virus cores. The kinetics of ATP incorporation by the vaccinia virus cores in the RNA polymerase and TrT assays are presented in Fig. 1. The rate of ATP incorporation in the presence of all four triphosphates under RNA polymerase assay conditions was usually 5 nmol per h per ODU vaccinia virus and proceeds linearly for at least 1 h. With ATP as the only substrate in the RNA polymerase reaction a more limited incorporation was seen. These results are in complete agreement with previously reported values (11). When the virus cores were tested for ATP polymerization under TrT conditions, but without added oligonucleotide, very little reaction was observed (cf. Fig. 3). The addition of $rA(pA)_5$ produced a remarkable stimulation of ATP incorporation, which continued in a linear fashion for at least 1 h. The rate of ATP incorporation was between 30 and 50 nmol per h per ODU of purified vaccinia virus. The addition of the other three ribonucleotide triphosphates to the TrT reaction resulted in a 60% inhibition of ATP incorporation.

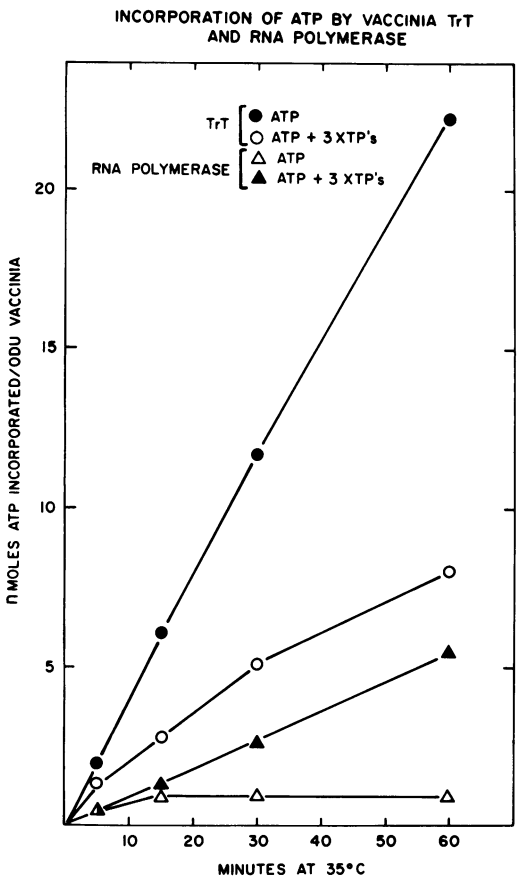

FIG. 1. *Incorporation of ATP in vaccinia TrT and RNA polymerase reactions. Purified vaccinia virus was assayed for TrT activity as described in the Materials and Methods* (●), *except that in addition to 0.5 mM ^{3}H-ATP the reaction mixture contained 0.4 mM GTP, 0.4 mM CTP, and 0.1 mM UTP* (○). *The RNA polymerase assay was as described in Materials and Methods, except that 0.5 mM ^{3}H-ATP (88 counts per min per pmol) was the labeled nucleotide* (▲), *and UTP, GTP, and CTP were omitted* (△).

Cosedimentation of virus and TrT activity. RNA polymerase activity and purified rabbitpox virus cosediment in sucrose density gradients (12). To insure that the TrT activity was virus associated and not the result of a cytoplasmic component with the same sedimentation properties as vaccinia virus, TrT activity was measured in cytoplasmic extracts of uninfected and ^{3}H-thymidine-labeled infected cells (Fig. 2). All of the ATP-polymerizing activity was associated with the ^{3}H-thymidine vaccinia virus near the center of the gradient. The uninfected cellular components and a small amount of virus material at the top of the gradients had no TrT activity.

Properties of the TrT activity. A comparison of the utilization of each of the ribonucleoside triphosphates by vaccinia TrT showed that ATP only was incorporated into an acid-insoluble product at a linear rate (Fig. 3). UTP and dATP were utilized to a very limited extent (less than 4% of the ATP polymerizaton rate), while CTP or GTP incorporation was not detected at the specific activities used.

Table 1 lists the inhibition of ATP polymerization by the addition of other nucleoside triphosphates. The TrT was more strongly inhibited by GTP than by either CTP or UTP. The degree of inhibition found with vaccinia TrT is comparable to earlier findings with calf thymus TrT (20). The degree of inhibition of the vaccinia TrT by dATP was quite remarkable.

The initiator and metal ion requirements of vaccinia TrT are shown in Fig. 4. No activity was detected in the absence of added metal ion, and Mn^{2+} was 30 times more effective than Mg^{2+} for TrT activation. The Mn to Mg activity ratio for calf thymus TrT is only 5 (20). The Mn^{2+}-dependent TrT reaction was greatly stim-

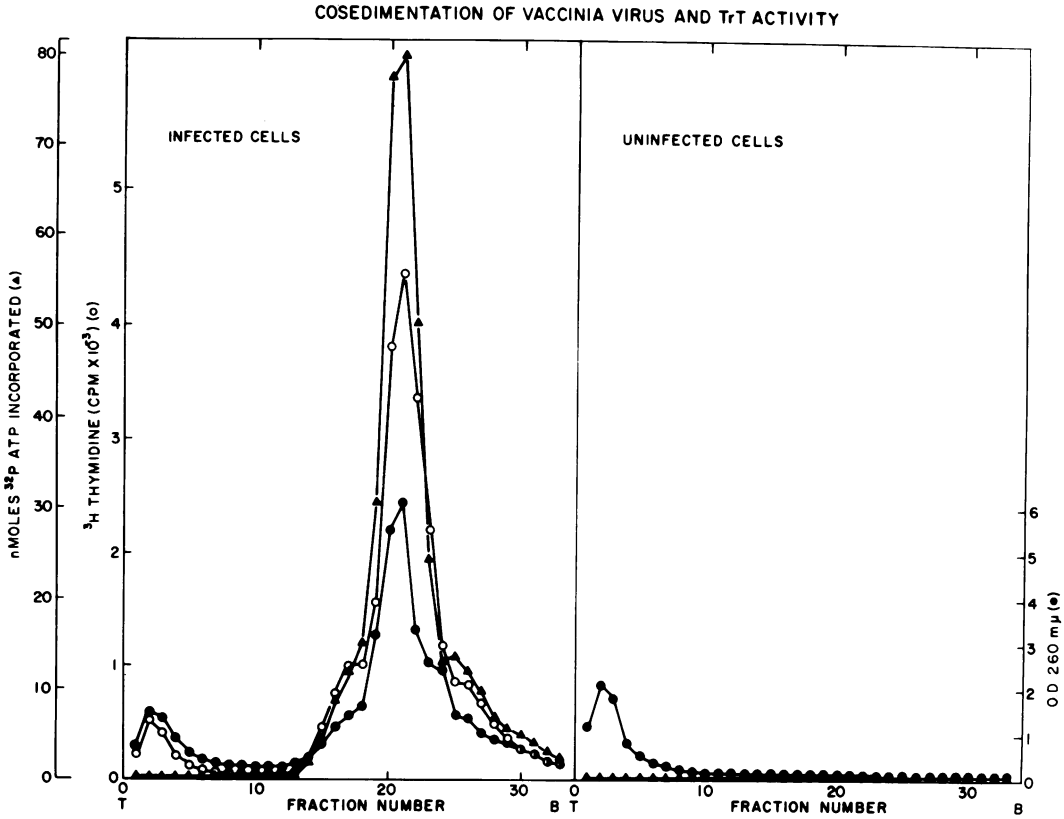

FIG. 2. *Cosedimentation of vaccinia virus and TrT activity. Cytoplasmic extracts from uninfected cells and from cells infected with vaccinia virus in the presence of ^{3}H-thymidine (0.5 μCi/ml) were sonically treated, pelleted twice through 36% (wt/vol) sucrose, and banded in a 25 to 45% (wt/vol) linear sucrose gradient as described by Kates and McAuslan (12). A sample from each fraction of the gradient containing ^{3}H-vaccinia virus was treated with Triton X-100 and mercaptoethanol and assayed for acid-insoluble radioactivity (○). Samples from gradients of vaccinia-infected cell extracts and uninfected cell extracts were assayed for TrT activity as described in Materials and Methods, except that 0.5 mM α^{32}P-ATP (48 counts per min per pmol) was used. TrT activity is expressed as nanomoles of ATP incorporated per 30 min per fraction (▲). Optical density at 260 nm was measured on each fraction (●).*

ulated by the addition of $rA(pA)_5$. A longer oligoribonucleotide, $rA(pA)_{12}$, initiated equally well (not shown). In the presence of Mg^{2+} the reaction proceeded slowly with or without an initiator. The uninitiated reaction in the presence of Mg^{2+} was probably the same ATP-polymerizing activity reported by Kates and Beeson (11) and shown in Fig. 1. The dependence of the TrT reaction on Mn^{2+} and added oligoribonucleotide serves to distinguish this activity from RNA polymerase which utilizes the vaccinia DNA template and is Mg^{2+} dependent (12).

Evidence for independent RNA polymerase and TrT activities. The investigations reported above suggest that the TrT and RNA polymerase may be separate activities, but further proof is desirable. Additional evidence comes from inhibitor studies, temperature inactivation curves, and pH activity profiles.

Poly A synthesis by vaccinia RNA polymerase was reported to be inhibited by the intercalating dyes ethidium bromide and proflavine sulfate but to be resistant to actinomycin D (11). A comparison of the effect of these drugs on vaccinia TrT and RNA polymerase is shown in Table 2. The vaccinia TrT was completely resistant to ethidium bromide, proflavine sulfate, and actinomycin D at concentrations which inhibit RNA polymerase completely. It is interesting to note that, although TrT was not inhibited by proflavine sulfate at 10 μg/ml, it was completely inhibited at 100 μg/ml. RNA polymerase activity was completely inhibited at either concentration of proflavine sulfate.

The activity of vaccinia RNA polymerase and TrT at several pH values is shown in Fig. 5. Maximum RNA polymerase activity was reached at pH 9.0 (12), whereas TrT activity was optimal around pH 8.0. Perhaps the most

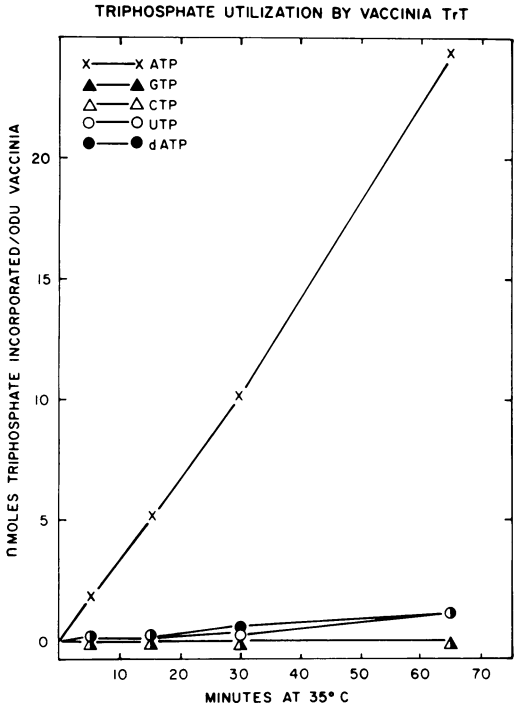

FIG. 3. *Ribonucleoside triphosphate incorporation by vaccinia TrT. The TrT reaction was as in Materials and Methods (x) except that the ^{3}H-ATP was substituted by 0.5 mM ^{3}H-GTP, 25 counts per min per pmol (▲); 0.5 mM ^{3}H-CTP, 93 counts per min per pmol (△); 0.5 mM ^{3}H-UTP, 18 counts per min per pmol (O); or 0.5 mM ^{3}H-dATP, 26 counts per min per pmol (●).*

TABLE 1. *Inhibition of TrT polymerization of ATP by other nucleotides*[a]

Nucleotide added	Polymerization rate[b]	Inhibition (%)
None	37	0
GTP	24	35
CTP	31	16
UTP	29	22
3 NTPs	18	51
dATP	3	92

[a] The TrT reaction mixture was as in Materials and Methods except that the concentration of ^{3}H-ATP was 0.25 mM (88 counts per minute per picomole). Where indicated, 0.25 mM nucleotide or a mixture of equal amounts of GTP, CTP, and UTP at 0.25 mM total nucleotide was added.

[b] Expressed as nanomoles per hour per ODU of vaccinia.

convincing evidence for separate enzymatic activities is seen in the temperature inactivation curves illustrated in Fig. 6. At 45 C, TrT activity remained constant for at least 1 h, whereas RNA polymerase was inactivated at a fairly rapid rate. This provides evidence that the activities are associated with different polypeptide chains and are therefore independent activities.

DISCUSSION

The ATP-polymerizing activity in purified vaccinia virus appears to be distinct from the RNA polymerase also present in vaccinia virions. Several properties differentiate this activity from RNA polymerase. Vaccinia TrT utilizes ATP as the only substrate, with linear incorporation of ATP into an acid-insoluble product for 1 h. ATP polymerization of TrT is inhibited by the presence of the other three nucleoside triphosphates, and the enzyme will not utilize UTP, CTP, or GTP alone. Incubation of vaccinia cores with ATP as the only substrate under RNA polymerase assay conditions results in limited ATP polymerization (11; Fig. 1). The reaction becomes linear upon addition of UTP, CTP, and GTP, and the product formed is

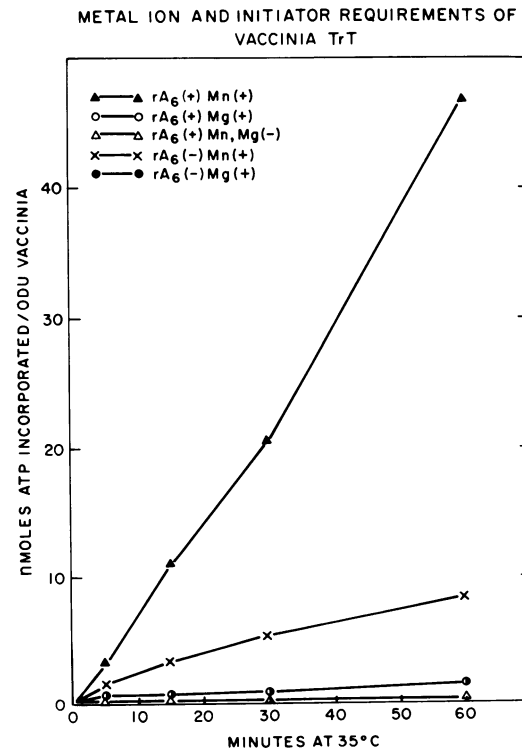

FIG. 4. *Metal ion and initiator requirements of vaccinia TrT. The TrT reaction mixture was as in Materials and Methods (▲), except as follows: 4 mM $MgCl_2$ was substituted for $MnCl_2$ (O); no metal ion was used (△); $rA(pA)_5$ was ommited (x); and $rA(pA)_5$ was omitted and 4 mM $MgCl_2$ was substituted for $MnCl_2$ (●).*

TABLE 2. *Drug inhibition of TrT and RNA polymerase*[a]

Drug added	TrT		RNA polymerase	
	Polymerization rate[b]	Inhibition (%)	Polymerization rate[b]	Inhibition (%)
None	30.2	0	3.9	0
Ethidium bromide				
100 μg/ml	31.2	0	0	100
Proflavine sulfate				
10 μg/ml	29.9	0	0	100
100 μg/ml	0.3	100	0	100
Actinomycin D				
5 μg/ml	34.3	0	0.1	97.4
50 μg/ml	27.7	8.6	0	100

[a] TrT and RNA polymerase assays were carried out as described in Materials and Methods except that the drugs indicated were added to the reaction mixtures.

[b] Expressed as nanomoles per hour per ODU of vaccinia.

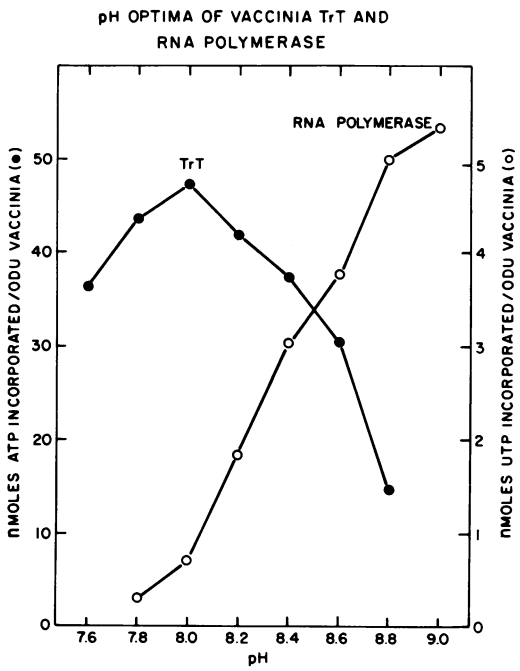

FIG. 5. *pH optima of vaccinia TrT and RNA polymerase. The TrT (●) and RNA polymerase (○) reaction mixtures were as described in Materials and Methods except that Tris-hydrochloride was used at the pH indicated.*

complementary to vaccinia DNA (11). The TrT and RNA polymerase also have different metal ion requirements (12), different pH optima, and different heat stabilities.

Vaccinia TrT and RNA polymerase also differ in their requirement for an initiator or template. An adequate demonstration of the TrT activity requires the addition of an exogenous oligoribonucleotide, presumably to act as an acceptor for AMP residues. No template seems to be required. The RNA polymerase presumably transcribes from the endogenous vaccinia DNA, because addition of other templates has no effect. The use of added oligonucleotide initiators permits a demonstration of the effect of inhibitors that is independent of transcription. The results with actinomycin D and the intercalating dyes indicate that poly A synthesis by TrT is not affected by the standard inhibitors of transcription. The reason for inhibition of TrT at high concentrations of proflavine sulfate is not known, but the results obtained with all three drugs and vaccinia TrT are in complete agreement with inhibitor studies with purified

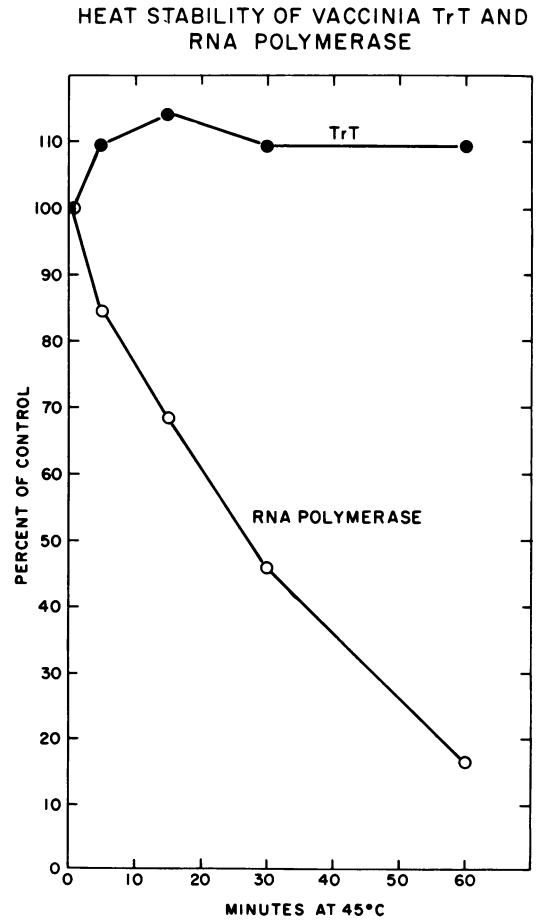

FIG. 6. *Heat stability of vaccinia TrT and RNA polymerase. Purified vaccinia virus was converted to cores as described in Materials and Methods and placed in a 45 C water bath. Samples were removed at the time intervals indicated and assayed for TrT activity (●) or RNA polymerase activity (○).*

TrT from calf thymus gland. The calf thymus TrT, which is free of contaminating nucleic acids, is also resistant to proflavine sulfate at 10 μg/ml and completely inhibited at 100 μg/ml (unpublished results).

Poly A sequences have been demonstrated in most messenger RNA molecules isolated from eukaryotic cells and animal viruses (1, 4, 7, 10, 13, 16, 18). Current evidence indicates that the addition of poly A to messenger RNA is a posttranscriptional event in both cellular and viral systems (4, 14, 16) and that poly A may be necessary to produce a functional message (16).

The biological function of TrT is not known, but one might expect that the enzyme responsible for poly A addition to early vaccinia virus messenger RNA either exists in the infecting virion, or that a cellular enzyme associates with the virus early in infection. Evidence is presented here that a separate enzyme capable of carrying out this modification is an integral part of mature vaccinia virions.

The question of whether the vaccinia TrT is coded by the vaccinia DNA or is a cellular enzyme that becomes enclosed in the viral capsid during maturation is not answered by this investigation. Although the viral TrT has several properties in common with the TrT purified from calf thymus cytoplasm, we do not suggest that they are identical.

ACKNOWLEDGMENTS

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