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Mechanism of Poly(A) Synthesis by Vaccinia Virus

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Data are presented which indicate that vaccinia DNA does not contain poly(dT) sequences the size of poly(A) sequences (50 to 200 nucleotides in length) found in vaccinia RNA. A hybridization experiment and polyacrylamide gel electrophoresis and DEAE-Sephadex chromatography of pyrimidine tracts show that poly(dT) sequences can account for no more than 0.1% of vaccinia DNA. Ultraviolet irradiation (which causes thymine dimer formation) and phleomycin (which binds to thymidine) both inhibit RNA synthesis but not poly(A) synthesis by vaccinia cores. These data are consistent with a nontranscriptive mechanism for vaccinia poly(A) synthesis. Both trypsin and 50 C heat treatment inhibit RNA synthesis more than poly(A) synthesis by cores, suggesting that separate enzymes may be involved in these syntheses. When the rate of core RNA synthesis is reduced by lowering the UTP and GTP concentrations, the size of the poly(A) sequences increase. These and other data suggest that transcription is involved in the termination of poly(A) synthesis in cores. This might be due to the displacement of growing poly(A) chains by recently completed RNA 3′ termini which have not yet acquired poly(A) sequences.

Considerable evidence has been presented which suggests that vaccinia virus synthesizes poly(A) on a viral DNA template (8, 10). Vaccinia cores which can synthesize RNA that resembles vaccinia mRNA in both size and sequence (8, 9, 19) also synthesize poly(A) when provided with ATP as the sole nucleoside triphosphate. Proflavin and ethidium bromide, which intercalate in DNA, inhibit both poly(A) and RNA syntheses. This was taken as evidence that poly(A) is synthesized by a DNA-dependent mechanism. However, it was not demonstrated that in this case the effect of the drugs on poly(A) synthesis was actually due to interaction with the DNA.

Kates and Beeson (10) also reported that *E. coli* RNA polymerase synthesized poly(A) when incubated with native vaccinia DNA and ATP. This would be expected if vaccinia DNA contained poly(dT) sequences which functioned as templates for poly(A) synthesis. Thirdly, hybridization experiments showed that poly(A) and poly(U) each annealed to about 1% of vaccinia DNA, but neither the specificity of the reaction nor the size of the annealant regions of the DNA were determined.

Nevertheless, since vaccinia DNA contains about 4.8×10^5 bases, about 4,800 bases or 2,400 base pairs were involved in the annealing. This is sufficient to account for about 20 poly(dT) regions the length of vaccinia poly(A). Vaccinia DNA has enough bases to account for an estimated 150 to 300 genes, assuming one gene contains about 1,000 base pairs. There are clearly too few poly(dT) sequences for there to be one for each gene. The implications of this for the mechanism of the synthesis and attachment of poly(A) to RNA are unclear.

During an investigation of the role of these sequences in poly(A) synthesis, we accumulated data which indicate that there are far fewer poly(dT) sequences—perhaps none—than we had earlier estimated. Further investigations indicated that poly(A) and RNA might be synthesized by separate polymerases. The paucity of poly(dT) sequences suggested that poly(A) might be formed by the nontranscriptive repetitive addition of AMP to polynucleotide chains. It thus seemed of interest to determine what regulated the length of the growing poly(A) chains.

MATERIALS AND METHODS

Preparation of virus and viral cores. The WR strain of vaccinia virus was grown in HeLa cells and

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purified by a modification (9) of the method of Joklik (7). The virus was titered on monolayers of 9- to 10-day-old chicken embryo fibroblasts.

Vaccinia cores were prepared according to Sheldon, Jurale, and Kates (19).

Preparation of RNA. (i) Viral core RNA. The standard assay for RNA synthesis contained 5×10^{10} cores per ml in 50 mM Tris-hydrochloride (pH 8.5), 10 mM mercaptoethanol, 5 mM $MgCl_2$, 1.25 mM each of ATP, GTP, and CTP, and 50 μM [^{3}H]UTP (100 μCi/μM). In some experiments the concentrations of the nucleoside triphosphates and radioactive label of the labeled precursor were changed according to the purpose of the experiment; these differences will be noted in the text or in figure and table legends.

Poly(A) synthesis (10) was assayed in a reaction containing 5×10^{10} cores per ml, 50 mM Tris-hydrochloride (pH 8.5), 10 mM mercaptoethanol, 5 mM $MgCl_2$, and 125 μM [^{3}H]ATP (40 μCi/μM). Again, variations in the concentrations of ATP or in the type or amount of radioactive label used will be noted in the text or in figure and table legends. Both poly(A) and RNA syntheses were performed at 37 C. The reactions were terminated with 0.2% sodium dodecyl sulphate (SDS), 10 mM EDTA, and RNA or poly(A) was extracted with chloroform-phenol (17). The aqueous phase was made 0.5 M NaCl or 0.2 M KCl and two to three volumes of 95% ethanol were added. After storage at -20 C for at least 3 h the precipitated RNA was centrifuged for 30 min at 0 to 4 C at $25,000 \times g$. The RNA was processed further as appropriate for the relevant experiment.

(ii) Viral late RNA. Viral late RNA was prepared according to Sheldon et al. (21).

Assays for poly(A). (i) Ribonucleases. To quantify the amount of poly(A) present in AMP-labeled RNA, the RNA was heated to 90 C for 5 min, then cooled rapidly and digested with 2 μg of pancreatic ribonuclease per ml, 1 μg of T_1 ribonuclease per ml (both from Worthington Biochemical Corp.) in 10 mM Tris-hydrochloride, (pH 7.5), 0.3 M NaCl at 37 C for 30 min. The digest was then precipitated with 5% trichloroacetic acid. Since only poly(A) is resistant to ribonuclease digestion under these conditions, it constitutes the acid-precipitable material. Variations in this procedure were as noted in the text and legends.

(ii) Poly(dT)-cellulose chromatography. Poly(dT) was synthesized and immobilized on cellulose (Munktell) according to Gilham (6). The poly(dT)-cellulose was then poured into a glass column and washed extensively with 10 mM Tris-hydrochloride (pH 7.5), 0.12 M NaCl at 25 C.

RNA to be assayed for poly(A) content was loaded on the column in 10 mM Tris-hydrochloride (pH 7.5), 5 mM EDTA, and 0.12 M NaCl at 25 C. The column was then washed extensively under the same conditions. Only RNA that contains poly(A) will remain on the column (8). To elute the poly(A)-containing RNA, the column bed temperature was elevated to 37 C and the column was developed with 10 mM Tris-hydrochloride (pH 7.5), 1 mM EDTA. The eluted RNA was usually concentrated by ethanol precipitation.

(iii) Preparation and use of poly(U) filters. Poly(U) filters were prepared and used according to Sheldon, Jurale, and Kates (19).

Preparation of DNA. Vaccinia DNA was isolated from vaccinia cores (9). A typical preparation consisted of 5×10^{11} to 10×10^{11} cores. The cores were suspended in 2 ml of SSC (0.15 M NaCl, 0.015 M sodium citrate) and were incubated with 1 mg of heat-treated Pronase per ml (Calbiochem) for 60 min at 37 C. After incubation with the heat-treated Pronase the preparation was made 1% SDS and incubated at 37 C for a further 60 min. The solution was then made 0.5 M $NaClO_4$ and shaken by hand for 30 min with 5 ml of chloroform-isoamyl alcohol (24:1, vol/vol). After recentrifugation the aqueous phases were pooled and the DNA was precipitated from two volumes of 95% ethanol. The ethanol precipitate was dissolved in 1 ml of 0.1× SSC. Overall recovery was greater than 90%.

Depurination of DNA. DNA was depurinated according to Burton (3). The DNA was first dialyzed against 2×1 liter of sterile, deionized, distilled water for 24 h each at 4 C. Subsequently, the aqueous DNA solution was incubated for 17 h at 30 C with three volumes of 88% formic acid, 3% diphenylamine. To extract the DNA, the solution was mixed with 0.5 volume of sterile deionized distilled water and six volumes of anhydrous ether and was shaken for 30 min. The aqueous phase was reextracted four times with anhydrous ether, then stored at -20 C until used.

Size analysis of polynucleotides. (i) DEAE-Sephadex column chromatography. Chromatography with DEAE Sephadex was used to fractionate oligonucleotides on the basis of chain length (12). DEAE Sephadex A-25 (Pharmacia) was equilibrated with 10 mM Tris-hydrochloride (pH 7.5), 7 M urea. The urea had previously been filtered through DEAE-cellulose equilibrated with 10 mM Tris-hydrochloride (pH 7.5). This filtration was necessary to remove a yellow contaminant in the urea.

A column with a bed volume of 2 ml was poured and washed with 100 ml of 10 mM Tris-hydrochloride (pH 7.5), 0.15 M NaCl, 7 M urea. The sample was then loaded in the same buffer, and eluted with 10 mM Tris-hydrochloride (pH 7.5), 7 M urea containing 0.3 M NaCl. After this step elution, a gradient elution of 48-ml volume between salt concentrations of 0.3 M and 0.55 M was performed. This was followed by a last elution of 1.0 M NaCl. The column was run at room temperature with a flow rate of 5 ml per h. Fractions of 1.5 ml were collected.

The fractions were assayed by adding three drops of an aqueous, saturated $BaCl_2$ solution and 10 ml of 95% ethanol, followed by 30 min of incubation on ice. The precipitate was then filtered through fiber glass filters (GF/C, 2.4 cm diameter), dried, and counted.

(ii) Polyacrylamide gels. The size of various polynucleotides was analyzed on 10% polyacrylamide gels as described by Peacock and Dingman (15) and Perry and Kelley (17).

RESULTS

Size of vaccinia poly(A). Vaccinia RNA was labeled with [^{3}H]adenosine late in infection

(after the start of DNA synthesis). Poly(A) was isolated from the RNA and analyzed by gel electrophoresis. Poly(A) from uninfected HeLa cell mRNA labeled for 2 h was prepared similarly as a control. The results are depicted in Fig. 1. Vaccinia poly(A) is heterogeneous, having estimated chain lengths from 50 to over 200 nucleotides. A large proportion of the species have chain lengths of 100 to 150 nucleotides. Because the HeLa [^{3}H]poly(A) ran as a moderately homogeneous band with an estimated chain length of 200 nucleotides, it is unlikely that the heterogeneity of vaccinia poly(A) is due to degradation during its isolation. The results of numerous other experiments (R. Sheldon, unpublished data) indicate that the heterogeneity of vaccinia poly(A) is not due to postsynthetic degradation. Rather, vaccinia poly(A) is synthesized in vivo as a collection of chains of heterogeneous lengths. Similar distributions to those seen in Fig. 1 are observed for poly(A) synthesized at various times throughout the infectious cycle.

Poly(A) was also isolated from RNA synthesized in vitro by cores, then fractionated by gel electrophoresis. The results shown in Fig. 8A show that this poly(A) is also heterogeneous, but is remarkably similar in size distribution to poly(A) from late RNA.

Role of DNA in the synthesis of poly(A) by cores. We investigated the role of the putative poly(dT) sequences in poly(A) synthesis by examining the effects of UV irradiation on the synthesis of poly(A) by cores. UV irradiation induces the formation of thymine-thymine dimers in DNA (25). The dimers inhibit the transcription of T7 DNA by *E. coli* RNA polymerase, probably by blocking the progress of the enzyme along the DNA duplex (4). If vaccinia cores synthesize poly(A) by transcribing a poly(dT) template, then UV irradiation should inhibit the synthesis of poly(A) as well as the synthesis of RNA. To test this, cores were irradiated for various periods of time, then assayed for their ability to synthesize RNA and poly(A). The results in Fig. 2 show that RNA synthesis was much more drastically inhibited by UV irradiation than was poly(A) synthesis.

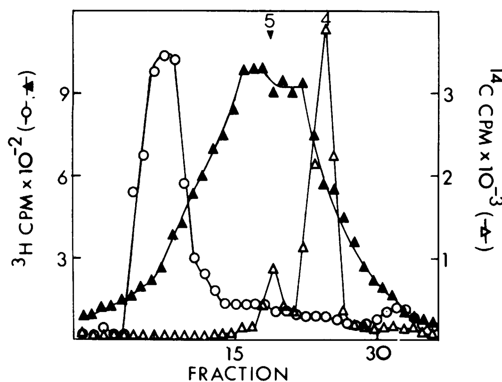

FIG. 1. *Size of vaccinia "late" poly(A). HeLa cells (5.6×10^7, 10^6/ml) were infected with vaccinia virus at 4 PFU per cell. At 180 min postinfection the cells were centrifuged and suspended in 25 ml of fresh medium containing 100 μCi of [^{3}H]adenosine. After 10 min the cells were collected and the cytoplasmic RNA was prepared. The RNA was precipitated from 2 M LiCl, then digested with 10 μg of T_1 ribonuclease. The poly(A) fragments were purified by poly(dT)-cellulose chromatography. HeLa poly(A) was prepared from 10^8 HeLa cells (10^6/ml) labeled with 100 μCi of [^{3}H]adenosine for 2 h, then isolated as described above. The poly(A) fragments and E. coli marker RNAs were fractionated on 6-cm 10% gels. The figure is a composite of two gels. Symbols: ○, HeLa [^{3}H]poly(A); ▲, vaccinia [^{3}H]poly(A); and Δ, E. coli 4 and 5S [^{14}C]-labeled RNAs.*

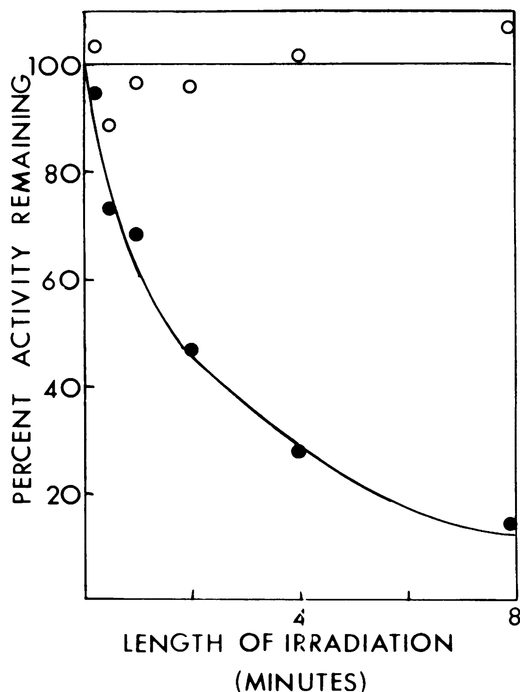

FIG. 2. *Inhibition by UV irradiation of core polymerase activities. Cores (10^{11}) in 0.56 ml of 50 mM Tris-hydrochloride (pH 8.5), 10 mM mercaptoethanol were irradiated at 4 C 40 cm from a 30W Sylvania germicidal lamp. At various times 50-μliter samples were removed and assayed for RNA and poly(A) synthesis as described in Materials and Methods. Symbols and incorporation in control reactions are: ○, poly(A), 2,707 counts/min; ●, RNA, 8,863 counts/min.*

This suggested that a DNA template might not be necessary for poly(A) synthesis.

Phleomycin was also used to approach this problem. Phleomycin binds specifically to thymidine (18) and inhibits DNA-dependent RNA synthesis (21). If poly(A) is not synthesized on a DNA template, then poly(A) synthesis should be much less sensitive to inhibition by phleomycin than is RNA synthesis. The results depicted in Fig. 3 confirm this prediction. Phleomycin at a concentration of 400 μg/ml inhibits virtually all RNA synthesis but barely affects poly(A) synthesis.

Both phleomycin and UV irradiation should inhibit poly(A) synthesis if it is DNA dependent. The lack of inhibition by either of these treatments suggests strongly that poly(A) is not synthesized on a DNA template.

Absence of poly(dT) sequences in vaccinia DNA. The probable DNA-independence of poly(A) synthesis led us to reinvestigate whether poly(dT) sequences were in fact present in vaccinia DNA. Hybridization experiments involving [^{3}H]poly(A) and vaccinia DNA immobilized to nitrocellulose membranes were performed in 50% formamide according to Marshall and Gillespie (11). The results are recorded in Table 1. No more [^{3}H]poly(A) bound specifically to filters containing 10 μg of vaccinia DNA than bound to blank filters. As a control, filters containing 0.1 μg of poly(dT) specifically bound an average of 1,942 counts/min of poly(A). Filters containing 0.01 μg of poly(dT) specifically bound an average of 183 counts/min of poly(A). Thus, it appears from this experiment that vaccinia DNA contains less than 0.01 μg/10 μg or 0.1% poly(dT) sequences. This conflicts with the results previously published (10) which indicated that vaccinia DNA contains about 1% poly(dT).

An alternative method of identifying poly(dT) sequences is to depurinate the DNA with formic acid-diphenylamine. This results in the hydrolysis of purine residues and the liberation of pyrimidine tracts (3). The pyrimidine tracts can then be isolated and characterized according to size and base composition.

Vaccinia DNA was labeled with [^{3}H]thymidine throughout the infectious cycle and purified from isolated virions as described in Materials and Methods. The [^{3}H]dTMP-labeled DNA was then depurinated (3). The depurination products were concentrated by ethanol precipitation and run on a 10% polyacrylamide gel. The results of this experiment are depicted in Fig. 4. Although there is considerable trailing from the major peak of low molecular weight

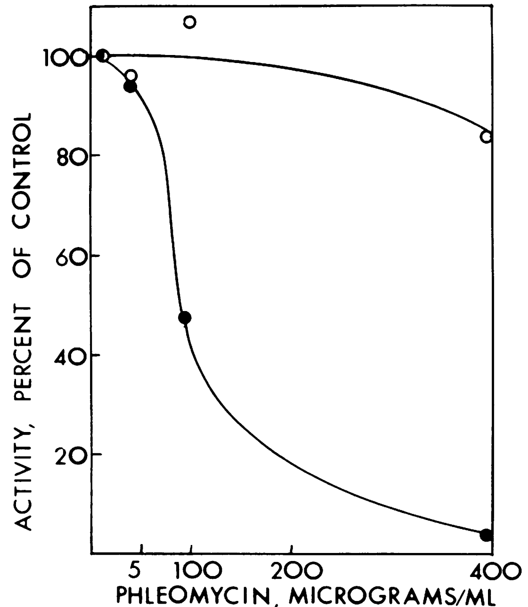

FIG. 3. *Inhibition by phleomycin of core polymerase activities. The reaction for each point contained, in 0.4-ml 10^{11} cores, the indicated concentration of phleomycin, 50 mM Tris-hydrochloride (pH 8.5), 5 mM $MgCl_2$, 10 mM mercaptoethanol, and either 0.125 mM [^{3}H]ATP (40 Ci/mol) to assay for poly(A) polymerase activity, or 1.25 mM each of ATP, GTP, CTP, and 50 μM [^{3}H]UTP (100 Ci/mol) to assay for RNA polymerase activity. After 15 min at 37 C the reactions were precipitated with 5% trichloroacetic acid and collected on GF/C filters. Symbols and incorporation in control reactions are: ○, poly(A), 7,473 counts/min; ●, RNA, 35,492 counts/min.*

material there is no identifiable peak in the region of migration of molecules 100 to 200 nucleotides in length. About 0.1% of the total loaded radioactivity was recovered from the 4*S* region of the gel.

The depurination products were also analyzed on a DEAE-Sephadex column. The results are shown in Fig. 5. This method is used to fractionate oligonucleotides on the basis of chain length (11). When the [^{3}H]dTMP-labeled depurination products of vaccinia DNA were applied to the column most of the material could be eluted with 0.3 M NaCl. A subsequent linear gradient of 0.3 to 0.55 M NaCl eluted virtually all of the remaining material. *E. coli* tRNA (about 75 to 80 nucleotides in length) labeled with ^{14}C was eluted with 0.45 M NaCl. Under similar conditions polio poly(A) (average chain length of 90 nucleotides) was eluted with 0.48 M NaCl (26). There was almost no [^{3}H]dTMP label eluting with the tRNA. Elution with 1.0 M NaCl did not release signifi-

TABLE 1. *Hybridization of [^{3}H]poly(A) to vaccinia DNA and poly(dT)*[a]

Material on filter	Counts/min bound	Avg. counts/min bound	Avg. counts/min minus background
Blank	580; 488; 690	586	0
10 ng of poly(dT)	762; 736; 809	769	183
100 ng of poly(dT)	2,769; 2,396; 2,420	2,528	1,942
10 μg of vaccinia DNA	646; 560; 539	582	−4

[a] [^{3}H]poly(A) was prepared in a 1.25-ml reaction which contained 0.2 U of polynucleotide phosphorylase per ml (Type b 15, P-L Biochemicals), 0.1 M Tris-hydrochloride (pH 8.5), 4 mM $MgCl_2$, 0.24 mM [^{3}H]ADP, and 1 μM pGpApG. After 2.4 h at 37 C the poly(A) was extracted and precipitated from ethanol. The poly(dT) used had an average chain length of 200 nucleotides. Nitrocellulose filters (Schleicher and Schuell, 2.4-cm diameter) containing the indicated amounts of polydeoxynucleotides were incubated with [^{3}H]poly(A) in 1 ml of 10 mM Tris-hydrochloride (pH 7.5), 3.4× SSC, 0.1% SDS, 50% formamide for 23 h at 34 C. Each filter was then rinsed in a beaker containing 500 ml of 2× SSC and 10 mM Tris-hydrochloride (pH 7.5), and each side was washed under suction with 50 ml of the same buffer. The filters were incubated for 12 h at 37 C in 1 ml each of 10 mM Tris-hydrochloride (pH 7.5), and 3.33× SSC containing 1 μg of deoxyribonuclease-free T_2 ribonuclease, swirled in 250 ml of 2× SSC, washed under suction with 100 ml each of 2× SSC, dried, and counted.

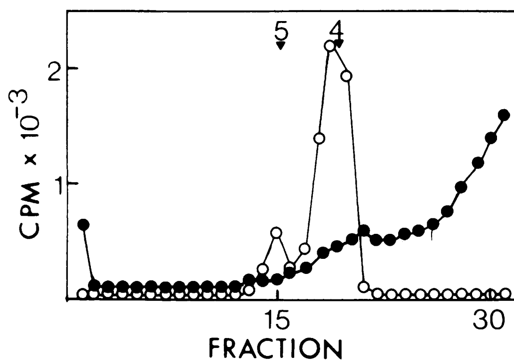

FIG. 4. *Gel electrophoresis of depurination products of [^{3}H]dTMP-labeled vaccinia DNA. [^{3}H]dTMP-labeled vaccinia DNA was purified and depurinated as described in Materials and Methods. A sample containing 7.5 × 10^5 ^{3}H counts/min was precipitated with ethanol and cofractionated on a 6-cm 10% gel with E. coli [^{14}C]UMP-labeled 4 and 5S RNA. Symbols: ●, [^{3}H]DNA; ○, [^{14}C]RNA.*

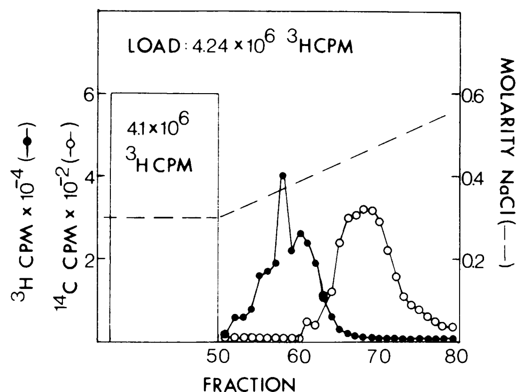

FIG. 5. *DEAE-Sephadex chromatography of depurination products of [^{3}H]dTMP-labeled vaccinia DNA. Depurination products (4.24 × 10^6 ^{3}H counts/min) and E. coli 4 and 5S RNA (3,000 ^{14}C counts/min) were applied to a DEAE-Sephadex column, eluted, and assayed as described in Materials and Methods. Symbols: ●, [^{3}H]DNA; ○, [^{14}C]RNA.*

cantly more material. Analysis by DEAE-Sephadex chromatography demonstrated that about 0.1% of the pyrimidine tracts eluted with, or later than, tRNA.

In summary, both hybridization and depurination experiments indicate that vaccinia DNA does not contain significant amounts of poly(dT) sequences the size of viral poly(A) sequences, in contradiction of an earlier report (11).

Distinction between poly(A) and RNA polymerases. It was of interest to determine whether poly(A) and RNA are synthesized by the same enzyme in vaccinia cores. One approach was to study the heat lability of the polymerase activities. Core RNA polymerase is unstable at 50 C (1, 14). Cores were incubated at 50 C for various periods of time, then assayed at 37 C for their ability to incorporate [^{3}H]UTP into RNA and [^{3}H]ATP into poly(A). The poly(A) reaction was carried out in the absence of CTP, GTP, and UTP. The results expressed in Fig. 6 show that RNA polymerase activity is more heat labile than poly(A) polymerase activity. RNA polymerase activity declines with a half-life of 5 min, compared to over 40 min for the poly(A) polymerase activity. Thus the polymerase activities are differentiable on the basis of their heat labilities.

A second approach was to follow the kinetics of loss of polymerase activities as a result of proteolytic degradation of the cores. Cores were incubated with trypsin for various lengths of time, diluted into 12-fold excess of ice-cold

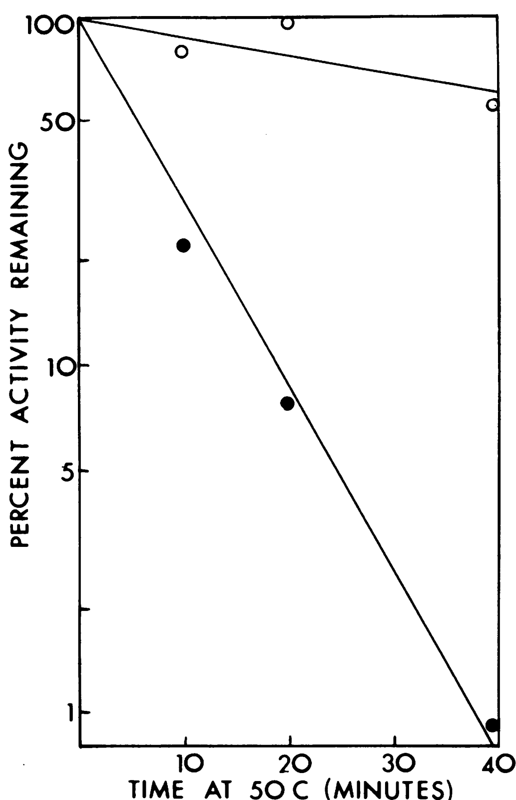

FIG. 6. *Heat labilities of core polymerase activities. Cores (2×10^{11} per ml) in 50 mM Tris-hydrochloride (pH 8.5), 10 mM mercaptoethanol were heated at 50 C. At the indicated times 50-µliter samples were cooled and assayed at 37 C for RNA and poly(A) syntheses as described in Materials and Methods. Symbols and incorporation in control reactions are:* O, *poly(A), 3,398 counts/min;* ●, *RNA, 13,971 counts/min.*

buffer and pelleted by centrifugation. The cores were then assayed for their ability to synthesize poly(A) and RNA as described above. The results shown in Fig. 7 demonstrate that poly(A) polymerase activity was significantly more resistant to proteolytic inactivation by trypsin than was RNA polymerase activity. This result demonstrates that the polymerase activities are also differentiable by their relative resistances to trypsin-mediated inactivation.

These findings are consistent with the notion that separate enzymes are responsible for the syntheses of RNA and poly(A) in viral cores.

Size of the poly(A) moiety varies with different RNA synthesis rates. Poly(A) polymerases isolated from other organisms (5, 21, 22, 23) add poly(A) to RNA in vitro by the repetitive addition of adenosine monophosphate to the 3′ ends of RNA. It is reasonable to assume that this is also the case in vaccinia cores. This raises the interesting question of what determines the length of the poly(A) moiety of core RNA molecules. It might be that the length of the poly(A) is determined by interaction of the nascent poly(A) with some structural component of the cores which serves as a gauge of poly(A) length. In this case the distribution of poly(A) lengths should be relatively invariant over a wide range of reaction conditions. Conversely, it might be that the length of the poly(A) is determined by the rate of RNA synthesis in the cores.

The rate of RNA synthesis in cores may be varied by varying the UTP and GTP concentrations (9). This changes the rate of elongation of nascent chains, but has no effect on the rate of initiation. We utilized this to determine the effect of varying the rate of RNA synthesis on the rate of poly(A) synthesis. Cores were incubated with 1.25 mM ATP and CTP, and either 1.25, 0.125, or 0.0125 mM UTP and GTP for 20 min at 37 C. RNA synthesis was measured as

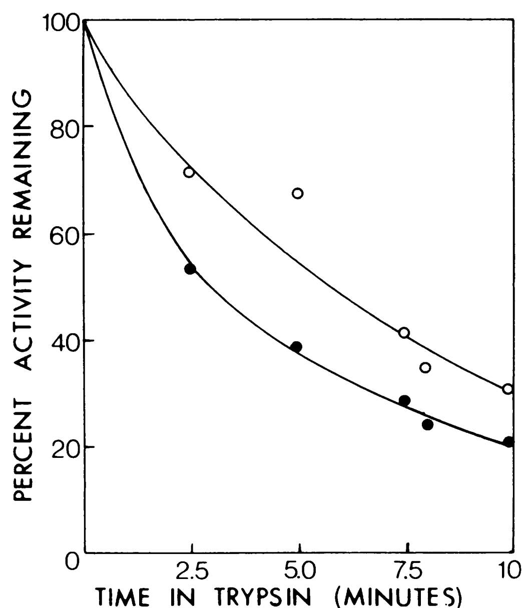

FIG. 7. *Trypsin inactivation of core polymerases. Cores (8×10^{10}) were incubated with 100 µg of trypsin in 0.4 ml of 0.05 M Tris-hydrochloride (pH 8.5), 10 mM mercaptoethanol at 37 C for the indicated times, diluted with 5 ml of ice-cold 0.05 M Tris-hydrochloride (pH 8.5), 10 mM mercaptoethanol, and immediately centrifuged at 0 C for 15 min at 25,000 × g. The pelleted cores were resuspended in 0.4 ml of the same buffer, and 0.1-ml samples were assayed for poly(A) and RNA polymerase activity as described in Materials and Methods. Symbols and incorporation in control reactions are:* O, *poly(A), 23,199 counts/min;* ●, *RNA, 27,182 counts/min.*

the rate of incorporation of [^{3}H]UTP into acid-precipitable material, whereas poly(A) synthesis was measured as the rate of incorporation of [^{3}H]ATP into ribonuclease-resistant, acid-precipitable material. The results are presented in Table 2. RNA synthesis is maximal in the presence of 1.25 mM UTP and GTP. In the presence of 1/10 and 1/100 this concentration of GTP and UTP, RNA synthesis proceeds at about 27 and 8%, respectively, of the maximal rate, whereas poly(A) synthesis proceeds at 72 and 41%, respectively, of its maximal rate. A diminution in the rate of RNA synthesis is accompanied by a concomitant but smaller diminution in poly(A) synthesis.

Core RNA contains an average of 1,000 nucleotides per molecule. By multiplying this by the ratio of (nanomoles of AMP in poly(A)/ nanomoles of nucleotides in RNA) one may estimate the average number of nucleotides in each poly(A) sequence. The estimates in the last column of Table 2 suggest that the length of the poly(A) moiety increases as the rate of RNA synthesis decreases. To test this the poly(A) sequences synthesized under different rates of RNA synthesis were isolated and electrophoresed in 10% polyacrylamide gels. The results in Fig. 8 demonstrate that the size of the poly(A) fragments increases as the rate of RNA synthesis decreases. Although it is difficult to accurately estimate average chain lengths from distributions as heterodisperse as those in Fig. 8, much of the poly(A) was about 100, 200, and 300 nucleotides in length when UTP and GTP concentrations were 1.25, 0.125, and 0.0125 mM, respectively.

A possible explanation for this phenomenon is that lower concentrations of UTP and GTP reduce the rate of extrusion of RNA from the cores as well as reduce the rate of RNA synthesis. This could result in the 3′ end of the RNA chain residing longer in the core, thereby being available longer as a primer for poly(A) polymerase. This in turn could result in longer poly(A) sequences.

This possibility was tested by determining the rates of extrusion of RNA from cores at 1.25 mM ATP and CTP, and either 1.25 or 0.0125 mM GTP and UTP. Cores were first incubated in the presence of 1.25 mM ATP, GTP, and CTP and 0.05 mM [^{3}H]UTP to establish a pool of [^{3}H]RNA within the cores. The cores were then diluted 100-fold into 1.25 mM ATP and CTP, 25 μg of actinomycin D per ml, and either 1.25 or 0.0125 mM GTP and CTP. Under these conditions cores do not synthesize RNA but do extrude about 60% of the RNA originally within the cores. The extrusion of the RNA was followed according to Kates and Beeson (9) as described in the legend to Fig. 9. The results depicted in Fig. 9 show that about 60% of the RNA was extruded under either condition. In the absence of net synthesis the rate of extrusion in the presence of 1.25 mM GTP and UTP is only a little faster than in the presence of 0.0125 mM GTP and UTP. This minor difference in the rate of extrusion is unlikely to be responsible for the three- to fourfold difference in the size of the poly(A) sequence.

The data presented in Table 2 and Fig. 9 suggest that RNA synthesis might be involved in the termination of ongoing poly(A) synthesis on completed RNA primers. In a final experiment we determined the size of the poly(A) sequence synthesized after RNA synthesis is stopped. Cores were first incubated with all four triphosphates for 15 min, then centrifuged out of the reaction mix. This preliminary incubation permits poly(A) and RNA syntheses and extrusion. The cores were resuspended in buffer

TABLE 2. *Effect of varying [UTP, GTP] on poly(A) and RNA syntheses by cores*[a]

[UTP, GTP] mM	RNA synthesized, (nmol)	Percent of maximum	Poly(A) synthesized (nmol)	Percent of maximum	Predicted avg. length of poly(A) (nucleotides)
1.25	7.1	100	0.46	100	65
0.125	1.9	26.7	0.33	71.7	167
0.0125	0.6	8.4	0.19	41.3	316

[a] Cores were incubated with 50 mM Tris-hydrochloride (pH 8.5), 5 mM $MgCl_2$, 10 mM mercaptoethanol, 1.25 mM ATP, 1.25 mM CTP, 0.1 mg of creatine phosphokinase per ml, 10 mM creatine phosphate and either 1.25 mM, 0.125 mM, or no UTP and GTP. The synthesis of RNA in nanomoles is calculated from the incorporation of [^{3}H]UMP into acid-precipitable material, knowing that UMP comprises about 33% of core RNA. The synthesis of poly(A) is calculated directly from the incorporation of [^{3}H]AMP into ribonuclease-resistant, acid-precipitable material. Poly(A) (0.06 nmol) was synthesized in the absence of UTP and GTP. This has been subtracted from the values for poly(A). The predicted average chain length of poly(A) was calculated assuming that there are an equal number of RNA and poly(A) chains, and that the average chain length of RNA is 1,000 nucleotides.

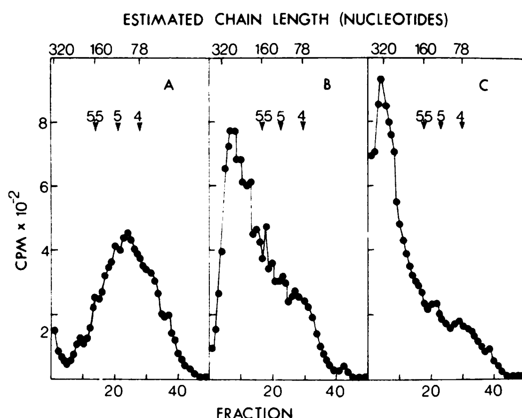

FIG. 8. *Gel electrophoresis of poly(A) synthesized in the presence of various concentrations of UTP and GTP. Cores (10^{11}) were incubated in 2 ml with 50 mM Tris-hydrochloride (pH 8.5), 5 mM $MgCl_2$, 10 mM mercaptoethanol, and 1.25 mM ATP and CTP at 37 C for 5 min. Then 50 µCi of [^{3}H]ATP was added and the reaction was made 1.25 mM (panel A), 0.125 mM (panel B), or 0.0125 mM (panel C) GTP and UTP. After 10 min the fractions were made 0.5% SDS, 10 mM EDTA, extracted with chloroform-phenol, precipitated from ethanol, and again from 2M LiCl. In a control experiment, poly(A) sequences up to at least 500 nucleotides long (synthesized by polynucleotide phosphorylase) remained in the LiCl supernatant. The pelleted RNA was digested with deoxyribonuclease, and T_1 and pancreatic ribonucleases and extracted. The poly(A) fragments were purified by poly(dT)-cellulose chromatography and were fractionated by electrophoresis on 10% gels. HeLa [^{14}C]UMP-labeled 4, 5, and 5.5S RNAs were run in the same gels. Their positions in the gels at the end of electrophoresis are marked in the panels. The chain length estimates should be viewed with caution, because conformational differences between poly(A) and the marker RNAs could alter their respective electrophoretic mobilities. Also, molecules are inadequately resolved in the top (about the first 10 fractions) of the gel.*

and one-half was added to [^{3}H]ATP and CTP, GTP, and UTP, while the other half was added to [^{3}H] ATP and CTP only. RNA synthesis is permitted in the first reaction but not the second. After 30 min of synthesis the RNA was isolated and precipitated with 2 M LiCl. Poly(A) was isolated by T_1 ribonuclease digestion of the RNA. Only poly(A) which had been attached to RNA is detected under these conditions (R. Sheldon, unpublished data). About two-thirds as much poly(A) was synthesized in the absence of RNA synthesis as in the presence. If RNA synthesis is necessary for the termination of poly(A) synthesis on completed RNA primers, then the poly(A) formed in the second reaction should be longer than that formed in the first reaction. The results presented in Fig. 10 demonstrate that this is the case. Poly(A) synthesized in the presence of concomitant RNA synthesis has an average chain length of about 130 nucleotides, whereas much of the poly(A) synthesized in the absence of concomitant RNA synthesis is greater than 350 nucleotides in length. This is consistent with but does not prove the notion that ongoing RNA synthesis is involved in the termination of poly(A) synthesis on preformed primers. A hypothesis accounting for these data will be presented in the Discussion.

DISCUSSION

Absence of poly(dT) sequences in vaccinia DNA. Experiments were performed to determine the role of DNA in the synthesis of poly(A)

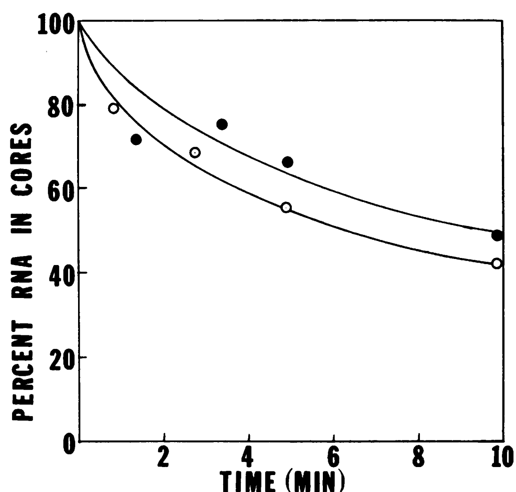

FIG. 9. *Effect of varying [UTP, GTP] on extrusion of RNA from cores. Cores (1.4 × 10^4) were incubated in 2.8 ml of 50 mM Tris-hydrochloride (pH 8.5), 5 mM $MgCl_2$, 10 mM mercaptoethanol, 1.25 mM each of ATP, GTP, and CTP, 50 µm of [^{3}H]UTP (40 Ci/mol) for 15 min at 37 C, then diluted 100-fold in 280 ml of 50 mM Tris-hydrochloride (pH 8.5), 5 mM $MgCl_2$, 10 mM mercaptoethanol, 1.25 mM each of ATP and CTP, and 25 µg of actinomycin D per ml. Two 20-ml samples were passed immediately through nitrocellulose filters and washed with 30 ml each of the Tris-$MgCl_2$-mercaptoethanol buffer. They were then incubated with 20 µg of pancreatic ribonuclease (to digest RNA outside the cores) in 1 ml of Tris-$MgCl_2$-mercaptoethanol buffer for 30 min at 37 C, then rinsed with 30 ml of buffer, 20 ml of 5% trichloroacetic acid, dried, and counted. Two 80-ml samples of the diluted reaction were made 1.25 mM UTP and GTP and 0.0125 mM UTP and GTP and incubated at 37 C. At the indicated times, 20-ml samples were removed from each and treated as above. Symbols and radioactivity in the control reactions are: ●, 0.0125 mM UTP and GTP, 6,667 counts/min; ○, 1.25 mM UTP and GTP, 6,671 counts/min.*

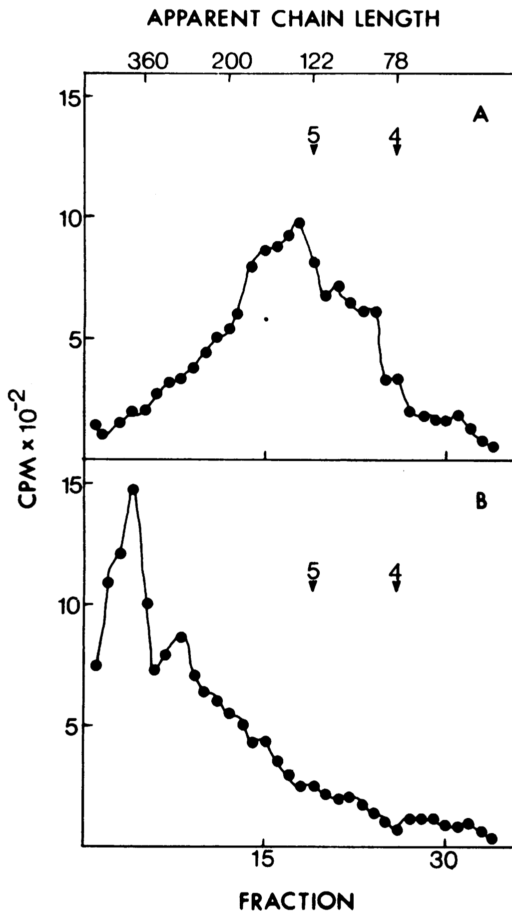

FIG. 10. *Effect of blocking transcription on poly(A) size. Core reactions were carried out as described in Materials and Methods. However, after centrifugation the cores were suspended in two reactions: the first contained all four NTPs (panel A), and the second contained only ATP and CTP (panel B). After 30 min at 37 C the RNA was extracted and [³H]poly(A) was prepared as described for Fig. 8.*

by vaccinia cores. Treatment with UV irradiation or phleomycin severely depressed RNA synthesis but had little or no effect on poly(A) synthesis. The simplest explanation of the different effects of such diverse agents on poly(A) and RNA synthesis is that only RNA synthesis is dependent on a DNA template.

Although Kates and Beeson (10) showed that proflavin sulfate and ethidium bromide inhibit core poly(A) synthesis, they did not show that the effect of the drugs was to bind to DNA and thus block the presumably transcriptive synthesis of poly(A). In fact, the calf thymus poly(A) polymerase, which synthesizes poly(A) in the absence of DNA, is also inhibited by proflavin sulfate (1). Thus the drugs might have been acting directly on the poly(A) polymerase protein.

This prompted a reinvestigation of the presence of poly(dT) sequences in vaccinia DNA. The results of hybridization experiments and an analysis of the pyrimidine tracts of vaccinia DNA revealed that there were no poly(dT) sequences the size expected (100 to 150 nucleotides in length) were they to code for vaccinia poly(A). Such tracts accounted for less than 0.1% of vaccinia DNA, a total of 240 bases, or enough for only one to three poly(dT) sequences the length of vaccinia poly(A).

These data directly contradict the earlier hybridization study (10), which indicated that poly(dT) sequences accounted for about 1% of vaccinia DNA. What could cause such a discrepancy? Kates and Beeson (10) did not measure the stability of the annealing product nor the size of the annealed regions in the hybridization experiments. It is possible that they observed nonspecific annealing of poly(A) to AT-rich regions in the DNA, or the poly(A) may have annealed to short regions of oligo(dT). We have not examined whether there are oligo(dT) tracts of, say, 10 to 20 nucleotides in length in vaccinia DNA.

Analysis of the RNA polymerase preparation used by Kates and Beeson (10) by slab gel electrophoresis (B. Polisky, personal communication) revealed that this particular preparation of enzyme contained numerous minor contaminants. Also, the intactness of the DNA templates was not checked. Poor templates and enzymic contaminants might account for the observed homopolymeric syntheses.

Vaccinia poly(A) polymerase. The simplest hypothesis about the mode of vaccinia poly(A) synthesis is that poly(A) is formed by the repetitive addition of adenosine monophosphate to the 3′ ends of RNA. Enzymes which catalyze this reaction have been isolated from a variety of organisms and, more recently, from vaccinia (13).

The question arose as to whether poly(A) and RNA were synthesized by the same enzyme in vaccinia cores. Figures 6 and 7 demonstrate that the RNA and poly(A) polymerase activities are differentiable on the basis of sensitivities to inactivation by heat and trypsin. Similar results were obtained by Brown, Dorson, and Bollum (1). These results could reflect the existence of separate enzymes responsible for RNA and poly(A) syntheses; that poly(A) is synthesized by a heat-resistant subunit of the RNA polymerase; or that the same enzyme

might be responsible for RNA and poly(A) syntheses. Whether the enzyme synthesizes RNA or poly(A) would depend on the association of the enzyme with different proteins in the viral core. Studies with a purified vaccinia poly(A) polymerase (13) may clear up the points in question.

Model for poly(A) synthesis and attachment to RNA in viral cores. It was shown that reduction in the concentrations of UTP and GTP depressed both poly(A) and RNA synthesis by cores. RNA synthesis was maximal in the presence of 1.25 mM UTP and GTP. In 0.125 and 0.0125 mM UTP and GTP, RNA synthesis proceeded at 27 and 8%, respectively, of the maximal rate. As the rate of RNA synthesis decreased the average length of the poly(A) sequence increased from 100 to 200 and 300 nucleotides, respectively. This cannot be explained by a variation in the rates of extrusion of RNA from cores.

A simple model which explains these results is as follows. The core DNA-dependent RNA polymerase transcribes a gene until it reaches a termination signal. At this point the 3' end of the completed RNA chain binds to a poly(A) polymerase—perhaps a distinct enzyme, perhaps a subunit of the RNA polymerase. Poly(A) is then added on to the 3' end of the RNA. When transcription of a second RNA chain is completed the 3' end of this chain binds to the poly(A) polymerase, displacing the growing poly(A) chain of the first RNA molecule. Addition of poly(A) to the second RNA molecule continues until it, in turn, is displaced by a third RNA molecule.

When RNA synthesis is slowed by reducing the UTP and GTP concentrations there will be fewer 3' termini passing through the poly(A) polymerase site per unit time. Thus a decrease in RNA synthesis will cause an increase in the size of poly(A) sequences.

This model predicts that diminution in the rate of RNA synthesis should have no effect on the rate of poly(A) synthesis. But, poly(A) synthesis is reduced 28% when RNA synthesis is reduced 73%. It might be that the synthesis of some poly(A) chains prematurely terminates for unknown reasons and that the total number of premature terminations increases with time. When RNA synthesis is slowed there will be a longer average time between the arrival of two successive 3' termini at each poly(A) polymerase site. A reduction in RNA synthesis would be accompanied by more premature terminations in poly(A) synthesis. This would be reflected in a decrease in the total rate of poly(A) synthesis.

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