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Ribonucleic Acid Synthesis in Vaccinia Virus

II. Synthesis of Polyriboadenylic Acid

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Vaccinia virus cores catalyze the incorporation of ATP into polyriboadenylic acid sequences of approximately 150 nucleotides in length. This incorporation of ATP occurs also in the presence of all four natural ribonucleotides in the reaction mixture. Poly A sequences are found in high molecular weight RNA synthesized by cores and evidence is presented for a covalent association between the poly A sequences and the regular viral RNA moiety.

The transcription of poly dT sequences in vaccinia DNA appears to be the likely source of poly A, since about 1% of vaccinia DNA contains regions which hybridize with synthetic poly A and poly U. In addition, the synthesis of poly A is inhibited by proflavin sulfate and ethidium bromide, which complex with DNA. Poly A sequences are also synthesized *in vivo* during the growth cycle of vaccinia virus in HeLa cells.

1. Introduction

It has been proposed that clusters of pyrimidines in the DNA may serve as recognition signals for DNA-dependent RNA polymerase (Szybalski, Kubinski & Sheldric, 1966). Certain synthetic polyribonucleotides form complexes selectively with one of the two strands of denatured DNA from a variety of organisms (Opara-Kubinska, Kubinski & Szybalski, 1964). The basis of the interaction between the synthetic ribopolymers and the separated DNA strands is presumed to be hydrogen bonding between complementary base pairs (Szybalski *et al.*, 1966). Phage T7 DNA can be resolved into two complementary strands after complexing with poly IG. Of particular relevance to transcription is the fact that the strand which complexes with the poly IG codes for more than 99% of the T7 messenger RNA synthesized *in vivo* (Summers & Szybalski, 1968). This suggests that clusters of dC in T7 DNA may be involved in the process of strand selection by the RNA polymerase.

No direct evidence linking pyrimidine clusters in the DNA with the transcription process is presently available. Nor has the question been answered concerning the possible transcription of pyrimidine clusters and the association of purine sequences with messenger RNA.

In this communication we present experimental evidence in support of the hypothesis that vaccinia virus DNA contains dAdT clusters which are transcribed as poly A sequences that are usually attached to high molecular weight viral RNA.

2. Materials and Methods

(a) *Virological procedures, chemicals*

The growth and purification of virus, the preparation of cores, conditions for core RNA synthesis and detection, purification of viral DNA and RNA, and sucrose gradient centrifugation were as previously described (Kates & Beeson, 1970). Proflavin sulfate and ethidium bromide were purchased from Cal-Biochem (Los Angeles, Calif.) and [^{3}H]poly U and [^{3}H]poly A were obtained from Miles Laboratories. Actinomycin D was a gift from Merk, Sharpe and Dohme (Rahway, N. J.).

(b) *Ribonuclease assay for poly A*

Reaction mixtures containing poly A were made 0·01 M with respect to EDTA and 0·2% with sodium lauryl sulfate. They were incubated at 37°C for 10 min with 40 μg pancreatic RNase/ml. and 12 μg T_1 ribonuclease/ml. The reaction mixture was then precipitated with trichloroacetic acid, filtered and counted.

(c) *RNA synthesis with vaccinia DNA and* Escherichia coli *RNA polymerase*

RNA polymerase was purified from *E. coli* B cells (Grain Processing Corp., Muskateen, Iowa) (Chamberlin & Berg, 1962). Reaction mixtures for poly A synthesis contained: Tris–HCl, pH 8·0, 40 μmoles; $MgCl_2$, 4 μmoles; 2-mercaptoethanol, 1·2 μmoles; ATP, 0·04 μmole (sometimes CTP, GTP and UTP were each present at a concentration of 0·5 μmole per assay); vaccinia DNA, 10 μg; *E. coli* polymerase, 20 μg protein. The total volume of the reaction mixture was 0·4 ml. and incubation at 37°C for 15 min.

(d) *Formation of DNA–RNA hybrids*

Hybrids of poly A and vaccinia DNA were formed using *E. coli* RNA polymerase (Chamberlin & Berg, 1964*a*) and heat-denatured vaccinia DNA in reaction mixture identical to that described above. After 20 min when the reaction had nearly gone to completion, the reaction mixture was made 0·5% with respect to sodium lauryl sulfate and was phenol extracted. The aqueous phase was precipitated overnight with 3 vol. of absolute ethanol from 0·5 M-NaCl. The nucleic acid was dissolved in sixfold SSC (SSC is 0·15 M-NaCl–0·015 M-sodium citrate, pH 7·0) adjusted to pH 4·5 with 0·01 M-acetate and treated for 20 min at 37°C with 10 μg T_2 ribonuclease/ml. (0·21 unit) (Bellamy & Joklik, 1967). The material was then filtered on a nitrocellulose membrane to collect the hybrid (Nygaard & Hall, 1964). This was then washed with 50 ml. of Tris–HCl, pH 7·4, 0·05 M containing 0·5 M-KCl. The filters were dried and counted by liquid scintillation.

Hybrids between vaccinia DNA and synthetic [^{3}H]poly A and [^{3}H]poly U were synthesized with 5 μg of denatured DNA immobilized on each nitrocellulose filter (Gillespie & Speigelman, 1965). The filters were incubated in twofold SSC at 30°C for 12 hr† with 500 μg of polyU (1 μc/10·9 μg). They were then treated with 12 μg of pancreatic RNase per ml. for 15 min at 37°C. The filters were washed and counted as previously described. For hybridization with ^{3}H-labeled poly A, the filters were incubated for 12 hr in twofold SSC at 50°C. They were then treated with 10 μg T_2 RNase/ml. for 20 min at 37°C, in sixfold SSC, pH 4·5, adjusted with 0·01 M-acetate. The filters were then washed and counted. [^{3}H]poly A (1 μc/23·4 μg) was present at a concentration of 500 μg/ml. in each 1-ml. hybridization tube. Thus amounts, greater by 100-fold, of the ribopolymers were used than the amount of vaccinia DNA in each of the hybridization tests.

(e) *Labeling of poly A synthesized* in vivo

Vaccinia-infected HeLa cells were labeled with [^{3}H]adenosine (13 c/m-mole; New England Nuclear) at a rate of 1 μc/ml. of culture. The infected cells were labeled in suspension culture for 10 min at 37°C. After this period the cells were diluted with ice-cold saline and centrifuged. RNA was prepared from the cytoplasmic fraction as previously

† Since the T_m of poly dArU is 45·2°C in 0·1 M-sodium ion, low temperatures were tested for hybridization of poly U with vaccinia DNA. 30°C was found to be optimum under the conditions employed. Similarly 50°C was found optimum for poly A (T_m rAdT = 64°C) hybridization with vaccinia DNA.

described (Kates & McAuslan, 1967). A similar procedure was used for labeling vaccinia RNA *in vivo* with [^{3}H]uridine.

3. Results

(a) *Time course of poly A synthesis by vaccinia cores*

Incubation of vaccinia cores with ATP results in the incorporation of ATP into an acid-insoluble polymer for a limited period of five minutes as shown in Figure 1, while

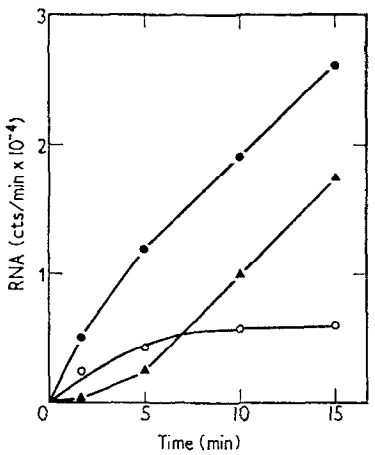

FIG. 1. The kinetics of incorporation of ATP into RNA by vaccinia cores.
All three reaction mixtures contained Tris–HCl 0·05 M, pH 8·5; MgCl, 5×10^{-3} M; 2-mercaptoethanol, 10^{-2} M; vaccinia cores, 2×10^{10} M. One reaction (—○—○—) contained ^{3}H-labeled ATP, $1{\cdot}25 \times 10^{-3}$ M (2 μc/μmole) as the only substrate. Another reaction mixture (—●—●—) contained CTP, GTP and UTP each $1{\cdot}25 \times 10^{-3}$ M in addition to the labeled ATP. The third reaction mixture (—▲—▲—) contained ^{3}H-labeled UTP $1{\cdot}25 \times 10^{-3}$ M (2 μc/μmole) plus a similar concentration of unlabeled ATP, CTP and GTP. Incubation was at 37°C for the times indicated on the axis.

incorporation of ATP in the presence of all four nucleotide substrates occurs for extended periods of time. In contrast to the incorporation of UTP into RNA by cores, ATP incorporation displays no initial lag period.

Table 1 indicates that ATP is the only one of the four nucleotides tested which is incorporated into a polymer when each was added individually to cores, and that this reaction was not sensitive to actinomycin D inhibition. On the other hand, incorporation of UTP into viral RNA was completely inhibited by actinomycin D.

In order to detect the synthesis of poly A sequences in the presence of messenger RNA synthesis, advantage was taken of the resistance of poly-purine ribopolymers to the action of pancreatic ribonuclease (Beers, 1960). Figure 2 shows the resistance of core poly A to pancreatic and T_1 RNases compared to the sensitivity of vaccinia core RNA labeled with [^{3}H]UTP in the presence of all four nucleotide substrates. The kinetics of incorporation of ^{3}H-labeled ATP into poly A sequences and into total RNA in the presence of all four nucleoside triphosphates is shown in Figure 3. Approximately 50% of the incorporated radioactivity was recovered in poly A sequences, and synthesis of poly A occurred over the entire 30-minute period.

TABLE 1

Incorporation of various nucleoside triphosphates into RNA by cores

Substrate(s)	$\mu\mu$moles incorporated
1. ATP	110
2. ATP + actinomycin D (25 μg/ml.)	98
3. GTP	0·8
4. CTP	0·7
5. UTP	0·5
6. ^{3}H-labeled UTP + ATP, CTP, GTP	109
7. ^{3}H-labeled UTP + ATP, CTP, GTP + actinomycin D (25 μg/ml.)	0·8

Each reaction mixture contained 2×10^{10} vaccinia cores, and 0·02 μmole of the indicated nucleoside triphosphate labeled with tritium (2 μc/assay). In 6 and 7, ATP, CTP and GTP were present at a concentration of $1{\cdot}25 \times 10^{-3}$ M. The reactions were incubated at 37°C for 20 min.

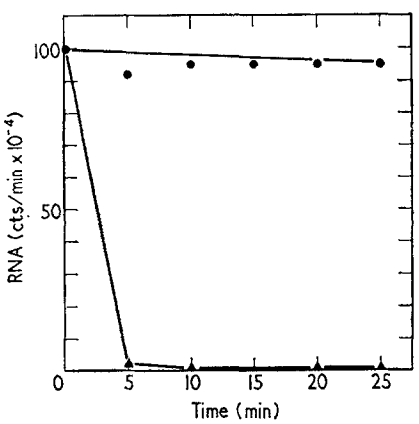

FIG. 2. Ribonuclease resistance of core poly A.

The kinetics of hydrolysis of ^{3}H-labeled poly A synthesized by cores and core messenger RNA labeled with [^{3}H]UTP were compared with 40 μg pancreatic RNase/ml. and 12 μg RNase T_1/ml. The core reaction mixtures containing either poly A or regular viral RNA were made 1×10^{-2} M with respect to EDTA and 0·2% with respect to sodium lauryl sulfate. They were then incubated at 37°C with the ribonuclease mixture and samples were assayed for amount of acid-insoluble radioactivity remaining at the indicated times. —●—●—, Poly A; —▲—▲—, viral messenger RNA.

(b) *Effect of intercalating dyes on poly A synthesis*

In order to test the contention that poly A synthesis is mediated by a DNA-dependent transcription mechanism, two intercalating dyes, proflavin sulfate and ethidium bromide, which inhibit bacterial RNA polymerase by complexing with the DNA (Richardson, 1966) were tested as inhibitors of poly A synthesis in vaccinia cores. The results shown in Table 2 indicate that concentrations of ethidium bromide and proflavin sulfate which effectively inhibit vaccinia core messenger RNA synthesis also inhibit substantially the incorporation of ATP into poly A. Actinomycin D, on the other hand, has no effect on poly A synthesis.

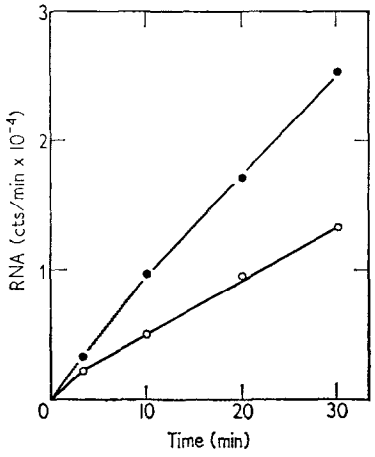

FIG. 3. Synthesis of poly A in the presence of all four ribonucleotide substrates.
The standard reaction mixture contained $1{\cdot}25 \times 10^{-3}$ M ^{3}H-labeled ATP, -UTP, -CTP and -GTP. Samples collected at the indicated times were split and one-half of each sample was treated with ribonuclease to determine the amount of poly A synthesized. —●—●—, Total RNA; —○—○—, RNase-resistant RNA.

TABLE 2

Drug inhibition of poly A synthesis and messenger RNA synthesis in cores

Drug	Messenger RNA		poly A	
	Radioactivity (cts/min)	Percentage inhibition	Radioactivity (cts/min)	Percentage inhibition
1. None	31,200	0	10,094	0
2. Proflavin (30 μg/ml.)	151	99·5	1042	89·5
3. Ethidium bromide (30 μg/ml.)	606	98	5600	44
4. Actinomycin D (30 μg/ml.)	23	~100	9670	4

Synthesis of messenger RNA labeled with [^{3}H]UTP was carried out by the standard core assay (Kates & Beeson, 1970) for 15 min at 37°C. Poly A synthesis was carried out in a similar reaction mixture except that ^{3}H-labeled ATP was the only substrate.

(c) *Synthesis of poly A with vaccinia DNA and* Escherichia coli *RNA polymerase*

In order to substantiate the hypothesis that poly A is the transcription product of dT sequences occurring in vaccinia DNA, the ability of the heterologous *E. coli* RNA polymerase to use vaccinia DNA as a template for poly A synthesis was tested and compared to T4 phage DNA. Native vaccinia DNA directed the synthesis of large amounts of poly A in the presence of the bacterial polymerase (Table 3). Poly A was made even in the presence of all four nucleotide substrates, thus ruling out a slippage mechanism which had been proposed for poly A synthesis by RNA polymerase with single-stranded DNA's (Chamberlin & Berg, 1964*b*). Native T4 DNA was a poor template for poly A synthesis resulting in tenfold less incorporation of ATP than

TABLE 3

Synthesis of poly A using vaccinia DNA and E. coli *RNA polymerase*

Substrates	Vaccinia DNA (cts/min)		T4 phage DNA (cts/min)	
	Regular	RNase treated	Regular	RNase treated
1. [^{3}H]ATP	6498	6122	600	589
2. [^{3}H]ATP (CTP, GTP, UTP)	57,920	6656	47,467	635

Both types of reaction mixtures contained 10 μg of native T4 or vaccinia DNA and 30 μg of purified *E. coli* polymerase. The ^{3}H-labeled ATP was present at a concentration of $1{\cdot}2 \times 10^{-4}$ M (1 μc/0·4 ml.) and the other nucleotide substrates, when used, were present at a concentration of 1×10^{-3} M. The reactions were incubated at 37°C for 10 min. Treatment with RNase was as described in Materials and Methods.

vaccinia DNA. Both DNA's were approximately equivalent as primers for regular RNA synthesis *in vitro*.

If the *E. coli* RNA polymerase transcribes the same dT clusters on vaccinia DNA that are presumably responsible for the synthesis of poly A by the endogenous polymerase present in cores, one would expect that the *E. coli* polymerase product would resemble in size the vaccinia core poly A. In order to answer this question, ^{14}C-labeled core poly A was sedimented in a sucrose gradient with ^{3}H-labeled poly-A synthesized from vaccinia DNA using the *E. coli* enzyme. It may be seen in Figure 4 that most of the poly A product synthesized by bacterial polymerase co-sediments with the poly A

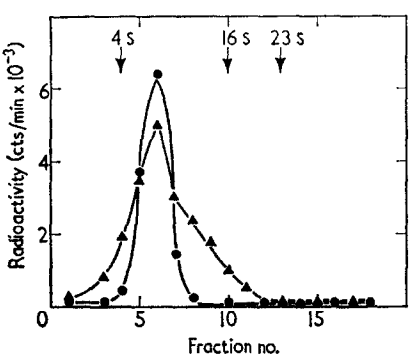

FIG. 4. Sucrose gradient distribution of poly A synthesized with *E. coli* polymerase and vaccinia DNA.

Vaccinia DNA, 10 μg, was incubated with *E. coli* polymerase, 20 μg protein, in a reaction mixture containing 0·1 M-Tris–HCl buffer, pH 8·0; 3×10^{-3} M-2-mercaptoethanol; 1×10^{-3} M-$MgCl_2$; and $1{\cdot}2 \times 10^{-4}$ M ^{3}H-labeled ATP (2 μc/mole). The product of this reaction was combined with ^{14}C-labeled poly A synthesized by vaccinia cores. After treatment with 0·5% sodium lauryl sulfate the RNA mixture was centrifuged through a 15 to 30% (w/w) sucrose gradient at 25°C for 16 hr in the Spinco SW27 rotor. The arrows indicate the position of *Bacillus subtilis* marker RNA components. —▲—▲—, *E. coli* polymerase ^{3}H-labeled poly A; —●—●— ^{14}C-labeled vaccinia core poly A.

synthesized by cores. The bacterial polymerase product, however, is somewhat more heterogeneous in size.

Since the *E. coli* RNA polymerase uses vaccinia DNA as a primer for poly A synthesis it was of interest to ask whether or not the latter enzyme also used this viral DNA as a primer for poly U synthesis. Table 4 shows the extent of incorporation of UTP and ATP into their respective ribopolymers. It is clear that native vaccinia DNA is an extremely poor primer for poly U synthesis compared to poly A synthesis. Denatured vaccinia DNA is somewhat better as a primer for poly U synthesis than native DNA, but there is still an appreciable preferential synthesis of poly A. CTP and GTP are also incorporated into ribopolymers to an appreciable extent by the bacterial polymerase using vaccinia DNA as primer. In this latter case the incorporation of CTP is about equal to the incorporation of GTP, and neither is incorporated more than 20% of the amount of ATP incorporated into poly A.

TABLE 4

Incorporation of single nucleoside triphosphates using vaccinia DNA and E. coli *RNA polymerase*

Reactants	Incorporated radioactivity (cts/min)
^{3}H-labeled ATP + native vaccinia DNA	10,747
^{3}H-labeled ATP + denatured vaccinia DNA	8695
^{3}H-labeled UTP + native vaccinia DNA	1557
^{3}H-labeled UTP + denatured vaccinia DNA	2837
^{3}H-labeled CTP + native vaccinia DNA	2537
^{3}H-labeled GTP + native vaccinia DNA	1593

Conditions were essentially identical to those described in Table 3 for incorporation of ^{3}H-labeled ATP.

(d) *Hybridization of poly A and Poly U with vaccinia DNA*

DNA–RNA hybridization provides a means by which to quantitate sequences in the DNA which are complementary to a given type of RNA. We therefore performed experiments to determine the percentage of vaccinia DNA which was complementary to poly A and poly U. Two basic types of experiment were carried out: (a) DNA–RNA hybrids were formed by the action of *E. coli* RNA polymerase using heat denatured vaccinia DNA as primer and ATP as the only substrate; (b) DNA–RNA hybrids using vaccinia DNA (heat-denatured) and synthetic radioactive poly A and poly U were formed by annealing at elevated temperatures.

It has been shown that transcription of single-stranded DNA by *E. coli* RNA polymerase results in the formation of a DNA–RNA hybrid (Chamberlin & Berg, 1964*a*). It may be seen from Table 5 that approximately 0·9% of the DNA was involved in a hybrid with poly A under these conditions. Since UTP was not incorporated effectively into poly U by the bacterial enzyme, the corresponding dA sequences in the viral DNA could not be determined by this method.

When synthetic ^{3}H-labeled poly A and ^{3}H-labeled poly U (Miles Biochemicals) were tested for their ability to hybridize with vaccinia DNA, it was found that at saturation, 0·7% of the DNA hybridized with poly A and 1·3% hybridized with poly U.

TABLE 5

Hybrid formation of poly A and poly U with denatured vaccinia DNA

	Incorporated radioactivity (cts/min)	Hybrid (cts/min)	μg RNA hybridized/ 10 μg DNA	Percentage DNA hybridized
1. ^{3}H-labeled ATP, *E. coli* polymerase and 10 μg Δdenatured vaccinia DNA	15,441	9500	0·09	0·9% ± 0·15%
2. ^{3}H-labeled poly A hybridized with 10 μg Δdenatured vaccinia DNA	—	—	0·07	0·7% ± 0·1%
3. ^{3}H-labeled poly U hybridized with 10 μg Δdenatured vaccinia DNA	—	—	0·13	1·3% ± 0·15%

In section 1 the RNA–DNA hybrid was synthesized by the action of *E. coli* RNA polymerase on heat-denatured vaccinia DNA in the presence of ^{3}H-labeled ATP as the only substrate in the reaction mixture (see Materials and Methods for details). The hybrid so synthesized was purified by treatment with 1% sodium lauryl sulfate and phenol extraction. It was then digested with 10 μg RNase T_2/ml. for 20 min at 37°C. The hybrid was then collected on a nitrocellulose filter (Nygaard & Hall, 1964), washed, and counted.

In sections 2 and 3 RNA–DNA hybrids were formed by annealing. Each mixture contained 5 μg heat-denatured vaccinia DNA immobilized on a nitrocellulose filter (Gillespie & Spiegelman, 1965) and 500 μg of either ^{3}H-labeled poly A (1 μc/23·4 μg) or ^{3}H-labeled poly U (1 μc/10·9 μg) in two-fold SSC. Hybridization was for 12 hr at 65°C and 30°C, for poly A and poly U, respectively. The filters containing the poly A hybrids were treated with RNase T_2 as described above, while the poly U hybrids were treated with 12 μg pancreatic RNase/ml. for 15 min at 37°C before washing and counting. Each datum is the average value of five annealing reactions.

The estimate of the amount of DNA complementary to poly U is likely to be high due to the possibility of forming triple-stranded structures of the type dArUrU (Chamberlin, 1965) and the undetermined RNase sensitivity of such structures.

In summary, both the *E. coli* polymerase results and the annealing data indicate that about 1% of the vaccinia DNA is complementary to either poly A or poly U. The above experimental data does not enable us to determine whether or not the sequences in the DNA are proximal to each other or are spread out over the entire viral genome.

(e) *Attachment of poly A sequences to viral RNA of high molecular weight in cores*

When ATP is the only substrate, cores synthesize a poly A product which is quite uniform in size, having a sedimentation coefficient of 4 to 5 s. In order to determine whether or not such poly A sequences were ordinarily attached to higher molecular weight messenger-like RNA, two types of experiments were performed: (a) cores were allowed to synthesize poly A from ^{3}H-labeled ATP as the only substrate and later the other three nucleotides were added and the radioactive ATP was chased by a 50-fold excess of unlabeled ATP. The subsequent attachment of the radioactive poly A sequences to larger molecular weight RNA was measured by sucrose gradient analysis of the reaction product. (b) Cores were incubated with all four nucleotides and with labeled ATP. The RNA product was tested for poly A content by the criterion of RNase resistance.

The results presented in Figure 5 show that poly A synthesized by cores in the presence of ATP alone does not subsequently become attached to high molecular weight viral RNA, nor does it increase in size during further incubation with ATP alone.

When cores were incubated with ^{3}H-labeled ATP and the other three nucleotides, radioactive ATP was incorporated into large molecular weight core RNA and also into

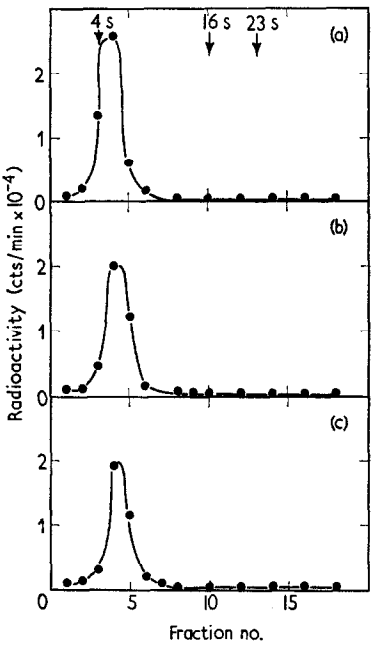

FIG. 5. Size distribution of core poly A showing lack of attachment to larger viral RNA synthesized after the poly A.

(a) Cores were allowed to synthesize poly A for 5 min with ^{3}H-labeled ATP as the only substrate at a concentration of $0{\cdot}625 \times 10^{-4}$ M (2 μc/ml.). A portion of the reaction mixture was then analyzed by sucrose gradient centrifugation.

(b) To a portion of the reaction described in (a) above, at 5 min, was added unlabeled ATP (final concentration 3×10^{-3} M) and incubation was continued for another 10 min. The product was analyzed by sucrose gradient centrifugation.

(c) A portion of the reaction described in (a) above, after 5 min, was incubated for an additional 10 min in the presence of 3×10^{-3} M-ATP, -CTP and -UTP, before centrifugation through the sucrose gradient. The arrows indicate the position of *B. subtilis* marker RNA components.

a component with the sedimentation coefficient of free poly A (4 to 5 s). Ribonuclease treatment of each gradient fraction revealed the existence of poly A sequences in higher molecular weight fractions of the gradient (Fig. 6(a)), in addition to a peak of poly A in the 4 to 5 s region. When cores were labeled with radioactive UTP little if any RNA of 4 to 5 s was observed and none of the RNA was RNase resistant (Fig. 6(b)).

(f) *Synthesis of poly A sequences in vaccinia-infected HeLa cells*

In order to rule out the possibility that synthesis of poly A by cores *in vitro* was an unnatural phenomenon resulting from conditions imposed by the experimenters, attempts were made to detect the synthesis of poly A in vaccinia-infected HeLa cells.

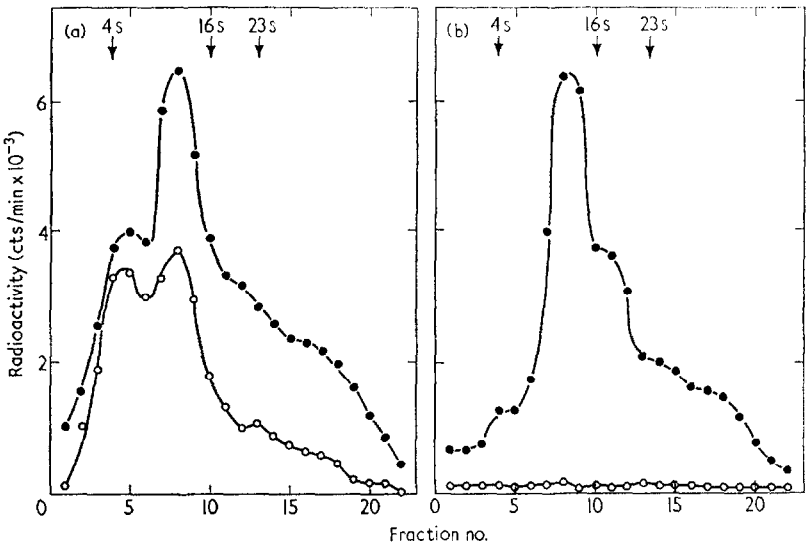

FIG. 6. Occurrence of poly A sequences in high molecular weight core RNA.

(a) Cores were incubated with ^{3}H-labeled ATP in the presence of the other 3 nucleoside triphosphates as described in the legend for Fig. 1. After 15 min at 37°C the product of the reaction was analyzed by sucrose gradient centrifugation. One-half of each fraction collected from the gradient was treated with RNase in order to determine the amount of poly A present. —●—●—, Without RNase; —○—○—, with RNase treatment.

(b) Cores were incubated with ^{3}H-labeled UTP in the presence of the other 3 nucleotide substrates for 15 min at 37°C, before sucrose gradient analysis, as in (a) above. —●—●—, Without RNase treatment; —○—○—, with RNase treatment. The arrows indicate the position of *B. subtilis* marker RNA components.

Infected cells were labeled at various times after infection with [^{3}H]adenosine for 10 minutes. (No detectable large molecular weight HeLa cell RNA enters the cytoplasmic fraction in this time (Becker & Joklik, 1964).) Then the cytoplasmic RNA was purified, and fractionated by means of sucrose gradient centrifugation. The existence of poly A was determined by RNase resistance after sucrose gradient fractionation. It may be seen from Figure 7(a) that an appreciable portion of the high molecular weight RNA which was labeled with radioactive adenosine at four hours postinfection was resistant to RNase. In contrast, infected cells labeled with radioactive uridine did not contain any RNase-resistant RNA (Fig. 7(b)). Uninfected HeLa cells which were labeled with [^{3}H]adenosine for 20 minutes did not contain any RNase-resistant RNA in the cytoplasmic fraction as shown in Figure 7(c).

Table 6 indicates the proportion of the [^{3}H]adenosine label incorporated into poly A as a function of time after infection of HeLa cells with vaccinia virus. The infected cells were labeled for ten minutes and the cytoplasmic RNA was purified and then treated with RNase to determine the percentage of poly A sequences (purification included a DNase digestion). It may be seen that from 9 to 16% of the incorporated adenosine occurred in an RNase-resistant polymer, presumably poly A, at all of the times tested. Poly A was also made in cells treated with FUdR from the time of infection. Since FUdR inhibits viral DNA synthesis (Salzman, Shatkin & Seebring, 1963), this experiment indicates that poly A is an "early" RNA. In fact, in cells infected in the presence of Streptovitacin, a potent inhibitor of protein synthesis, poly A

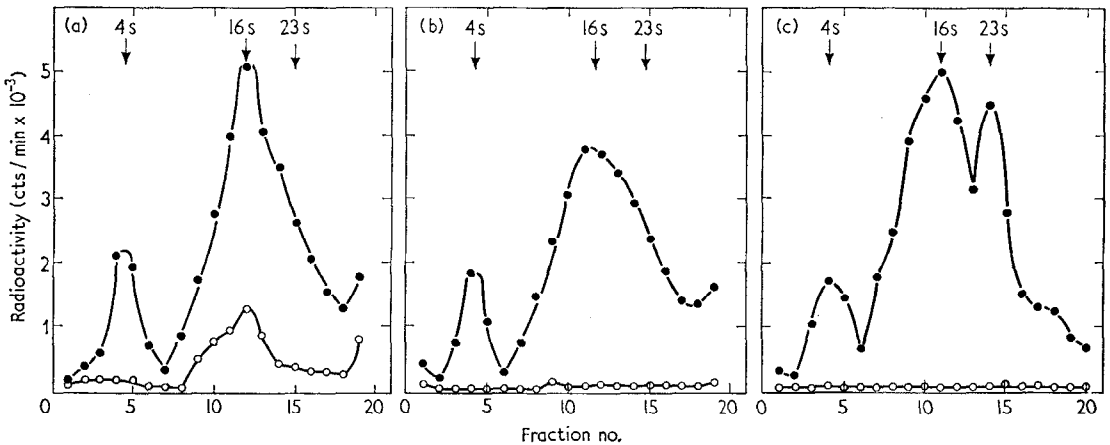

FIG. 7. Synthesis of poly A sequences in vaccinia-infected HeLa cells, and its association with high molecular weight viral RNA.

(a) Infected cells were labeled with [^{3}H]adenosine (1 μc/ml.; 13 c/m-mole) for 10 min at 4 hr postinfection. The RNA was purified from the cytoplasmic fraction (Kates & McAuslan, 1967) and analyzed by sucrose gradient centrifugation. Each fraction from the gradient was then tested for RNase-resistant poly A. —●—●—, Without RNase treatment; —○—○—, with RNase treatment.

(b) Infected cells were labeled for 10 min with [^{3}H]uridine instead of [^{3}H]adenosine and the RNA was analyzed as in (a) above. —●—●—, Without RNase treatment; —○—○—, with RNase treatment.

(c) Uninfected HeLa cells were labeled for 20 min with [^{3}H]adenosine. The cytoplasmic RNA was purified and analyzed by sucrose gradient centrifugation. —●—●—, Without RNase treatment; —○—○—, with RNase treatment. The arrows indicate the position of *B. subtilis* marker RNA components.

TABLE 6

Synthesis of poly A at various times in the vaccinia growth cycle

Labeling time (hr postinfection)	Drug	Total RNA radioactivity (cts/min)	RNase-resistant RNA, (cts/min)	Percentage RNase-resistant RNA
1	None	45,000	7300	16·2
2	None	76,000	8086	10·6
4	None	79,000	9825	12·4
6	None	52,000	5976	11·5
2	FUdR (time 0) 5×10^{-5} M	81,000	7676	9·5
2	Streptovitacin (time 0) 15 μg/ml.	62,000	5740	9·3
(Core ^{3}H-labeled UTP RNA control)		40,000	188	0·25
(Core ^{3}H-labeled poly A control)		7000	6567	93

Vaccinia-infected HeLa cells (300 particles/cell) were labeled with [^{3}H]adenosine at the indicated times postinfection. After the 15 min labeling period the RNA was purified from the cytoplasmic fraction and tested for RNase resistance as described in the legend to Fig. 2. The effect of FUdR (5×10^{-5} M) or Streptovitacin (15 μg/ml.), when added at the beginning of infection, on the synthesis of poly A sequences in viral-infected cells is also shown. Also included is a control for the effectiveness of the RNase digestion using [^{3}H]UTP-labeled, core, messenger RNA, synthesized in the standard core assay (Kates & Beeson, 1970); and a control for the resistance of core poly A to the ribonuclease digestion.

sequences are still synthesized. Under the latter conditions the infecting virus particles accumulate as cores in the cytoplasm of the infected cells and do not become uncoated (Dales, 1965; Kates & McAuslan, 1966).

(g) *On the nature of poly A attachment to larger viral RNA*

The stability of the association between poly A sequences and the higher molecular weight viral RNA was tested on RNA purified from infected cells which were labeled with [^{3}H]adenosine four hours postinfection (Fig. 8).

In one case the purified RNA was dissolved in 75% dimethyl sulfoxide and then heated to 80°C for ten minutes in the presence of 250 μg/ml. of unlabeled synthetic poly A. After rapid cooling in an ice bath the material was analyzed for the distribution of the radioactive poly A in a sucrose gradient. The poly A carrier was added to prevent possible reannealing of viral poly A to messenger RNA. It may be seen in Figure 8 that the size distribution of the poly A sequences did not change. Similarly, when the purified RNA was heated in 0·01 M-Tris–HCl buffer pH 7·4 for ten minutes at 95°C no change occurred in the sedimentation pattern of the poly A.

Treatment of the purified [^{3}H]adenosine-labeled RNA prepared from infected cells with pancreatic RNase before sucrose gradient centrifugation resulted in a dramatic change in the sedimentation of the poly A to a rather homogeneous peak of approximately 4 S as shown in Figure 9. It is not certain that this is a true reflection of the size of the poly A sequences found in the larger molecular weight RNA since even highly purified RNase results in a low level of endonucleolytic cleavage of poly A chains (Beers, 1960).

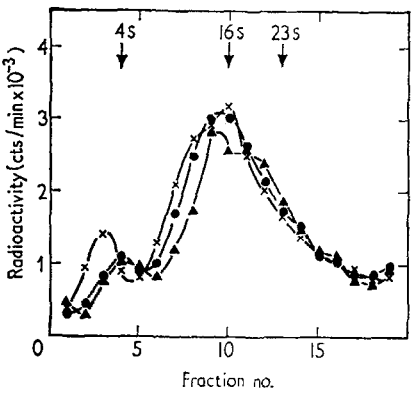

FIG. 8. Resistance of poly A attachment to high molecular weight RNA to heating and dimethylsulfoxide.

Purified, [^{3}H]adenosine-labeled RNA prepared from vaccinia-infected HeLa cells (see Fig. 7) was subjected to the following treatments: —●—●—, No treatment; —▲—▲—, heated to 95°C for 10 min in 0·01 M-Tris–HCl, pH 7·4, and rapidly cooled; —×—×—, dissolved in 75% dimethylsulfoxide in 0·01 M-Tris–HCl, pH 7·4, and heated to 80°C for 10 min in the presence of 250 μg of synthetic poly A (Miles Biochemicals)/ml. and then rapidly cooled. The 3 RNA samples were centrifuged on sucrose gradients and each fraction was then treated with RNase to determine its content of poly A. The arrows indicate the position of *B. subtilis* marker RNA components.

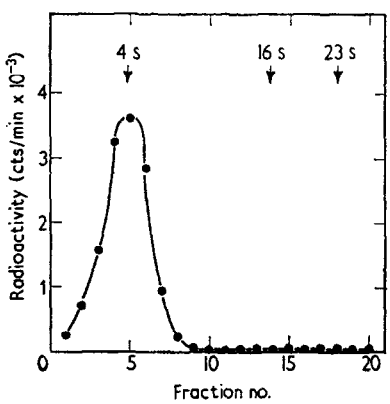

FIG. 9. Size distribution of poly A sequences attached to high molecular weight viral RNA synthesized *in vivo*. Infected-HeLa cell RNA labeled with [^{3}H]adenosine (see Fig. 7(a)) was treated with 40 μg pancreatic RNase/ml. and 12 μg RNase T_1/ml. for 15 min at 37°C. After phenol extraction to remove residual nuclease, the RNase-resistant RNA was analyzed by sucrose gradient centrifugation. The arrows indicate the position of *B. subtilis* marker RNA components.

4. Discussion

In this report we have presented experimental evidence that polyriboadenylic acid is synthesized by vaccinia virus cores *in vitro*, and during the viral growth cycle *in vivo*. Direct evidence in support of the hypothesis that the synthesis of poly A is mediated by a DNA–template mechanism was provided by the fact that two drugs which intercalate with DNA, ethidium bromide and proflavin sulfate, inhibit the synthesis of the ribopolymer. Indirect evidence that a DNA-mediated mechanism is involved in poly A synthesis was provided by the hybridization data which indicated that at least 1% of the vaccinia DNA is comprised of dAdT regions. Furthermore vaccinia DNA was a much better primer for poly A synthesis using *E. coli* RNA polymerase than was T4 phage DNA. As pointed out in the text, it is unlikely that the synthesis of poly A by the bacterial polymerase is due to a repeated copying of a small region of dT in the DNA by a slippage mechanism since the latter reaction is ordinarily inhibited completely by the presence of other nucleoside triphosphates in the reaction mixture (Chamberlin & Berg, 1964*b*), whereas the synthesis of poly A reported here was not at all inhibited.

The selectivity displayed by *E. coli* RNA polymerase in that it synthesized preferentially poly A but not poly U with vaccinia DNA as primer, deserves further comment. In this respect, the bacterial enzyme shows the same bias as the endogenous core polymerase, which also does not synthesize poly U. A pertinent question might be: does the bacterial polymerase display assymetric transcription of vaccinia DNA and does it only make messenger RNA copy the sense strand of the DNA? Unpublished experiments carried out in our laboratory indicate that the RNA synthesized by transcription of vaccinia DNA with *E. coli* RNA polymerase can be completed up to 95% in a DNA–RNA hybridization by unlabeled RNA prepared from vaccinia-infected HeLa cells. Furthermore, the RNA synthesized by the bacterial polymerase self-annealed to an extent of less than 8%. Thus it appears that the bacterial enzyme recognizes some signals on the vaccinia DNA which enable the enzyme to make the proper selection of strands. A similar situation has been recently reported for *E. coli* polymerase with Adenovirus-2 DNA and SV40 DNA (M. Green and H. Westphal, personal communications). It is possible that homopolymeric sequences in the DNA serve as effective binding sites for RNA polymerase and that the DNA strand containing the pyrimidine sequence is selectively transcribed (Szybalski *et al.*, 1966). The experimental evidence presented above, that the poly A sequences in vaccinia virus may be attached to viral messenger RNA by a covalent bond provides some support for the argument that the pyrimidine cluster on the DNA occurs on the same strand as the regions which are transcribed.

When ATP was added to cores in the absence of the other nucleotide substrates, only a limited amount of poly A synthesis occurred. Assuming a molecular length of about 150 nucleotides for the poly A, which has a maximum sedimentation of about 5 S, it can be calculated that approximately 200 molecules of poly A are synthesized per core before the reaction is terminated. But hybridization data indicates that only 1% of the viral DNA could code for poly A synthesis. This amount of dT sequences, if transcribed only once, would result in the production of only 31 molecules of poly A of the observed size. Thus it is likely, if a DNA–template mechanism exists for poly A synthesis, that the dT sequences are transcribed repeatedly in the absence of the other three triphosphates as substrates.

It should be pointed out that only about 14% of the transcribable sequences in vaccinia DNA are used by cores (Kates & Beeson, 1970). Thus the inability of cores to synthesize homopolymeric sequences other than poly A does not necessarily mean that these are not transcribed at any other time in the viral growth cycle. In fact, the finding that vaccinia DNA stimulates the incorporation of CTP into poly C and GTP into poly G using the *E. coli* polymerase may be indicative of the existence of dCdG regions on the DNA and these may be transcribed along with "late" viral RNA.

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Note added in proof: At the time of writing we were unaware of the discovery of AMP-rich RNA in Ehrlich ascites cells (Edmonds & Caramela, 1969). This RNA, which constituted 1% of the total cellular RNA, had a sedimentation coefficient of 8 to 10 s and contained more than 90% adenosine.

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