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Capped and Polyadenylated Low-Molecular-Weight RNA Synthesized by Vaccinia Virus In Vitro

ENZO PAOLETTI,* BERNARD R. LIPINSKAS, AND DENNIS PANICALI

Division of Laboratories and Research, New York State Department of Health, Albany, New York 12201

In the presence of ATP plus two other ribonucleoside triphosphates or in reactions containing all four ribonucleoside triphosphates and actinomycin D, vaccinia virus synthesizes in vitro discrete low-molecular-weight RNA molecules ranging in size from about 20 to several hundred bases. A novel feature of these small RNA molecules is that they are capped and methylated at the 5′ terminus, containing both ^{m}GpppGm and ^{m}GpppAm type cap structures, and in addition these molecules are polyadenylated at the 3′ terminus. Hybridization of these RNAs to restriction fragments derived from vaccinia virus DNA indicates a considerable degree of complexity, suggesting the presence of a large number of promoters throughout the genome. However, measurable sensitivity to pancreatic RNase of the 5′ capped end of these RNAs while in hybrid form to the DNA suggests other possible roles for these small RNAs in vaccinia virus mRNA biogenesis.

Our laboratory has described the synthesis of high-molecular-weight (HMW) RNA by vaccinia virus in vitro (23). This RNA has been partially characterized as follows. (i) The HMW RNA is virion associated. It can, however, be chased to monocistronic-sized (8 to 12S) mRNA which can be released from the virus (24). (ii) Its cleavage is dependent upon ATP and inhibited by ethidium bromide (24), and the RNA can be cleaved in vitro to monocistronic-sized fragments in the presence of an endoribonuclease solubilized from cores (27). (iii) The RNA is capped and methylated at the 5′ terminus but is not significantly polyadenylated at the 3′ terminus (22, 26). (iv) The HMW RNA can be translated in in vitro systems, and its translation products are essentially identical to the translation products of mature, released 8 to 12S mRNA. Further experiments indicated that the translated sequences were at or very near the 5′ end of the HMW RNA (6). (v) The transcriptional complexity of the HMW RNA was indistinguishable from that of virion-released mature monocistronic messages (25). (vi) The ability to detect the synthesis of HMW RNA was dependent on some as yet undefined parameter related to the concentration of ATP in the in vitro RNA polymerase reaction (26). A number of the observations regarding the synthesis of HMW RNA by vaccinia virus have been confirmed by other laboratories (12, 13).

An hypothesis that we proposed was that the virion-associated HMW RNA was a precursor to virion-released mature mRNA (24). The ability to synthesize preferentially this large RNA by limiting the ATP concentration (26) suggested a very useful system for studying a possible precursor-product relationship. When those experiments were pursued, we discovered further complexities in the system that would not allow a clear-cut definition of this hypothesis. This complicating factor was the observation of the synthesis of discrete low-molecular-weight RNA molecules by the virus in vitro. The synthesis and characterization of these small RNA molecules, which are capped and methylated at the 5′ terminus and polyadenylated at the 3′ terminus (polyadenylated leaders, or PALs), will be the subject of this communication.

MATERIALS AND METHODS

Cells and virus. The WR strain of vaccinia virus was purified from infected HeLa cells as previously noted (23).

RNA synthesis and analysis. Purified vaccinia virus was used to synthesize RNA in vitro, and the RNA was purified, analyzed on sodium dodecyl sulfate (SDS)-sucrose gradients, probed for polyadenylic acid [poly(A)] sequences on polyuridylic acid [poly(U)] GF/C filters or poly(U)-Sepharose chromatography, and analyzed for methylated constituents by procedures already described (22-26).

Purification of commercial ribonucleoside triphosphates. All four ribonucleoside triphosphates, purchased from P-L Biochemicals, were repurified by column chromatography as described by Hurlbert (14).

Electrophoretic analysis of PALs on polyacrylamide gels. PALs labeled either internally with [α-^{32}P]UTP or [α-^{32}P]CTP or at the 5′ terminus with *S*-[^{3}H]adenosylmethionine ([^{3}H]AdoMet) were analyzed on either cylindrical or slab gels according to the

procedure described by Maniatis et al. (16). The gels consisted of either 3.5% polyacrylamide for the analysis of ACU and actinomycin D (Actino D) PALs or 12% for the analysis of AGU and ACG PALs. Urea at a concentration of 7 M was present in the gel. PALs were purified by phenol extraction, desalted on columns of Sephadex G50, and chromatographed on poly(U)-Sepharose. After ethanol precipitation, the PALs were dissolved in 98% formamide, boiled for 2 min, and, after addition of dye markers, electrophoresed at a constant 120 V. Cylindrical gels were sliced into 2-mm sections, treated with H_2O_2 for 18 h, and counted. Slab gels were exposed to 10% acetic acid–40% methanol overnight, dried, and radioautographed, or were prepared for fluorography as described by Bonner and Laskey (4). The relative size of the PALs was determined by using 4S (85 bases) or 5S (120 bases) RNA markers from *Escherichia coli* (Miles Laboratories, Elkhart, Ind.) and the dye markers bromophenol blue and xylene cyanol$_{FF}$. On 12% polyacrylamide gels, the bromophenol blue dye migrates in the position of an oligonucleotide of 13 bases, and the xylene cyanol$_{FF}$ dye migrates similar to an oligonucleotide of 58 bases (16). On 3.5% polyacrylamide gels, the bromophenol blue and xylene cyanol$_{FF}$ markers migrate similar to molecules of approximately 100 and 175 bases, respectively.

DNA purification, restriction endonuclease digestion, and hybridization of PALs and RNA to restriction fragments. Virus was purified without freezing, and virions were lysed in 0.2 M sodium phosphate, 6 M urea, 0.01 M EDTA, 1% Sarkosyl NL 97, and 2% 2-mercaptoethanol, using 6 absorbancy units at 260 nm of virus per ml of this buffer, overnight at room temperature (9). DNA was extracted initially with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then repeatedly with chloroform-isoamyl alcohol (24:1), and dialyzed extensively against 10 mM Tris-hydrochloride (pH 7.6)–0.1 mM EDTA. DNA was concentrated by repeated extraction with 2-butanol (33) and redialyzed. Final DNA concentrations were adjusted to approximately 100 μg/ml.

Purified vaccinia DNA (15 μg) was digested with 20 U of *Hpa*II restriction endonuclease (Bethesda Research Laboratories, Bethesda, Md.) in 600 μl of 20 mM Tris-hydrochloride (pH 7.4), 1 mM dithiothreitol, and 7 mM $MgCl_2$ at 37°C for 5 h. The reaction was terminated by heating to 65°C for 5 min. A melted solution (100 μl) containing 2.4% agarose, 40% glycerol, 5% SDS, and 0.25% bromophenol blue was added, and the sample was layered in the liquid state onto a 1 to 2% agarose gradient gel (30 by 14.5 by 0.3 cm) in 36 mM Tris-hydrochloride (pH 7.8), 30 mM NaH_2PO_4, and 1 mM EDTA. Electrophoresis was in the same buffer at 4°C for 18 h at a constant 30 mA.

DNA in the gels was denatured in 0.9 M NaCl–0.5 M NaOH at room temperature for 1 h, followed by neutralization in 0.9 M NaCl–1.0 M Tris-hydrochloride (pH 7.4) for 1 h (30). The gel was then rinsed in water prior to transfer to a nitrocellulose sheet (Schleicher and Schuell BA85) overnight essentially as described by Southern (32).

Hybridization of PALs and RNA to DNA fixed to nitrocellulose was done as follows. Nitrocellulose filters were cut into 1.4-cm strips, placed on a backing of Whatman no. 1 filter paper, moistened with hybridization buffer 2× SSC (0.3 M NaCl and 0.03 M sodium citrate, pH 7.2) mixed with an equal volume of phenol-saturated 2× SSC (30), coiled, and placed in a 1-cm-diameter siliconized glass vial containing the appropriate radiolabeled PAL or RNA in 1.5 ml of hybridization buffer. After 20 h at 60°C each strip was washed once with 2× SSC, and the strip was cut into two 0.7-cm strips. Each section was then washed once in 35 ml of 2× SSC at 60°C for 1 h. One of the 0.7-cm strips from each pair was then incubated in 25 ml of 2× SSC for 1 h at room temperature either in the absence or presence of 20 μg of heat-treated pancreatic RNase per ml. After three 30-min washes in 2× SSC, the hybridization strips were taped to a sheet of filter paper, dried under vacuum for 15 min, and prepared for fluorography as described by Bonner and Stedman (5) by dipping the entire sheet into a solution of 0.4% PPO (2,5-diphenyloxazole) dissolved in 2-methyl-napthalene. Exposure was for 25 days.

Analysis of PALs and RNA by synthesis of cDNA. Vaccinia RNA or poly(A)$^+$ PALs were reverse-transcribed in the following reactions. Complementary DNA (cDNA) was synthesized using the avian myeloblastosis virus reverse transcriptase (100 U/ml) in reactions containing 50 mM Tris-hydrochloride (pH 8.4), 60 mM NaCl, 4 mM $MgCl_2$, 20 mM dithiothreitol, 0.6 mM deoxyribonucleoside triphosphates (dNTP's), 100 μg of poly(A)$^+$ PAL or RNA per ml, 20 μg of oligo(dT)$_{(12-18)}$ per ml, and 100 μg of Actino D per ml. cDNA from each of the PALs or RNA was synthesized using each of the four ^{3}H-labeled dNTP's in separate reactions. The concentration of the ^{3}H-labeled dNTP was reduced to 0.1 mM, and the specific activities were kept between 862 and 1,129 cpm/pmol. The cDNA product was analyzed at six time points from 5 min to 1 h, and the relative incorporation was used to deduce the complementary bases in the vaccinia PALs and RNA.

Nearest-neighbor analysis of [α-^{32}P]ATP-labeled PALs. PALs were synthesized as described above except that the concentration of [α-^{32}P]ATP was reduced to 1 mM. Nearest-neighbor analysis was performed exactly as described by Volckaert and Fiers (34). Radioautographs were scanned and quantified using an ACD-18 Automatic Computing Densitometer (Gelman Instrument Co., Ann Arbor, Mich.).

RESULTS

Synthesis of low-molecular-weight RNA by vaccinia virus in vitro. The ability to preferentially synthesize virion-associated HMW RNA by using vaccinia virus in vitro and depleting the reaction of ATP (26) indicated a reasonable method for determining whether a precursor-product relationship existed between this putative unprocessed-precursor RNA and virion-released mature mRNA. To assure ourselves that the only initiation of RNA that occurred was that seen in HMW RNA, the following experiment was done. Vaccinia virus was incubated in in vitro RNA polymerase reactions with ATP, CTP, UTP, [^{3}H]AdoMet, and

[α-^{32}P]GTP from 0 time. Other reactions contained [^{3}H]AdoMet from 0 time in the presence of ATP, CTP, and UTP. At 20 min after appropriate levels of ATP were depleted (26), [α-^{32}P]GTP was added, and RNA synthesis was continued for another 20 min. Using the methylation of the 5'-terminal capped structures as an indicator of initiation, the former conditions allow one to measure the ^{3}H/^{32}P in the monocistronic 8 to 12S mRNA. The latter allow measurement of the ^{3}H/^{32}P ratio in HMW RNA. Since GTP was present only after ATP was depleted in the latter reaction, the ratio of ^{3}H/^{32}P expected should be approximately one fourth of that observed in the standard reaction. This is based on the requirement for GTP for capping and subsequent methylation (19), on the approximate sizes of the RNA molecules involved (1,000 bases for the 8 to 12S mRNA and 4,000 bases for the HMW RNA), and on the information that these molecules are not methylated internally (22). Unexpectedly, the ^{3}H/^{32}P ratio observed in the ATP-depleted reaction was approximately three times greater than that under standard RNA synthesis conditions (Table 1), indicating a significantly greater number of initiation events than could be attributed to the synthesis of HMW RNA. Analysis of these reaction products by sedimentation analysis on SDS-sucrose gradients (Fig. 1) indicated that indeed the [α-^{32}P]GTP was incorporated into an HMW RNA sedimenting between the 18S and 28S rRNA markers. [^{3}H]AdoMet-labeled trichloroacetic acid-precipitable material, on the other hand, sedimented as a dominant peak of radioactivity near the 4S RNA marker. As shown by other experiments, this same incorporation of [^{3}H]AdoMet into low-molecular-weight RNA occurred in reactions where GTP was omitted completely. Since the essential role of GTP in the capping and methylation of vaccinia virus RNA has been rigorously shown by Moss and co-workers (10, 17, 19), our observation suggested that methylation occurred be-

TABLE 1. *Trichloroacetic acid-precipitable incorporation of [^{3}H]AdoMet and [α-^{32}P]GTP by vaccinia virus in vitro*[a]

Conditions of synthesis	[^{3}H]AdoMet	[α-^{32}P]GTP	^{3}H/^{32}P
Standard reaction	16,348	27,183	0.60
ATP-depletion reaction	29,129	15,867	1.84

[a] In the standard RNA polymerase reaction, [^{3}H]AdoMet and [α-^{32}P]GTP were present from 0 to 15 min. In the ATP-depletion reaction, [^{3}H]AdoMet was present from 0 to 40 min and [α-^{32}P]GTP was present from 20 to 40 min. Trichloroacetic acid-precipitable material was collected on Whatman GF/C filters. Appropriate corrections for isotope spill were made.

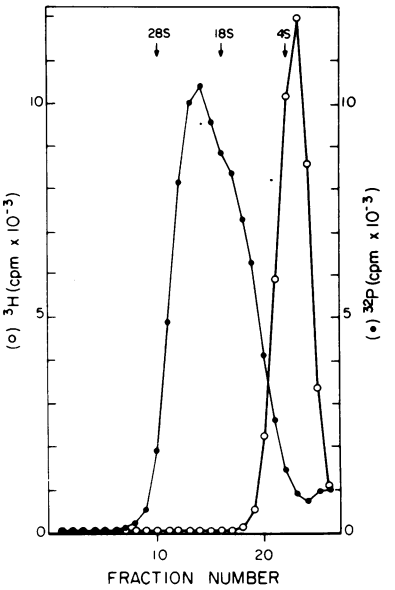

FIG. 1. *SDS-sucrose gradient analysis of products labeled with [^{3}H]AdoMet and [α-^{32}P]GTP by vaccinia virus in vitro. [^{3}H]AdoMet was present at 0 time in an in vitro reaction with vaccinia virus along with ATP, CTP, and UTP. After 20 min of incubation to allow ATP depletion, HMW RNA was labeled by the addition of [α-^{32}P]GTP. At 40 min the reaction was terminated, and RNA products were purified by phenol extraction, ethanol precipitated, and analyzed on 15 to 30% SDS-sucrose gradients. Trichloroacetic acid-precipitable material labeled with [α-^{32}P]GTP (●) or [^{3}H]AdoMet (○) was collected on Whatman GF/C filters. Appropriate corrections for isotope spills were made. The locations of rRNA markers run on parallel gradients are noted.*

cause GTP was present in our reactions, either as a contaminant introduced via one or more of the other ribonucleoside triphosphates, or endogenously, in the purified virions. This latter possibility will be commented upon later. To determine whether GTP was present as a contaminant in the other ribonucleoside triphosphates, we repurified each of the four ribonucleoside triphosphates and measured the ability of purified vaccinia virus to incorporate [^{3}H]-AdoMet as a function of the ribonucleoside triphosphates present in the reaction. As is demonstrated in Table 2, [^{3}H]AdoMet was incorporated into a trichloroacetic acid-precipitable material even after commercial triphosphates were repurified in our laboratory. Data of note from the above experiment are as follows. (i) Little or no methylation occurred in reactions containing one or two triphosphates. This is significant because it indicates that the methylation we observed is not capped and methylated poly(A). (ii) Significant levels of [^{3}H]AdoMet incorpora-

TABLE 2. *[^{3}H]AdoMet incorporation by vaccinia virus in vitro using either commercial or repurified ribonucleoside triphosphates*[a]

Ribonucleoside triphosphates	Commercial		Repurified	
	[^{3}H]AdoMet cpm	Relative to control	[^{3}H]AdoMet cpm	Relative to control
ATP	153	0.00	150	0.00
GTP	0	0.00	109	0.00
CTP	337	0.00	383	0.00
UTP	0	0.00	429	0.00
ATP+GTP	14,158	0.04	4,487	0.01
ATP+CTP	1,233	0.00	3,459	0.01
ATP+UTP	22,442	0.07	5,073	0.01
GTP+CTP	405	0.00	191	0.00
GTP+UTP	0	0.00	184	0.00
CTP+UTP	381	0.00	86	0.00
ATP+GTP+CTP	85,750	0.25	41,809	0.11
ATP+GTP+UTP	92,502	0.27	107,389	0.27
ATP+CTP+UTP	66,014	0.19	34,281	0.09
GTP+CTP+UTP	344	0.00	88	0.00
ATP+GTP+CTP+UTP	344,404	1.00	393,297	1.00

[a] [^{3}H]AdoMet incorporation (counts per minute; cpm) was measured as trichloroacetic acid-precipitable material collected on Whatman GF/C filters from in vitro reactions containing various ribonucleoside triphosphates as described.

tion were observed in the presence of three ribonucleoside triphosphates as long as ATP was present. (iii) Approximately 9% of the level of methylation observed with all four triphosphates was obtained without the addition of exogenous GTP. Although the level of methylation was reduced when repurified ATP, CTP, and UTP were compared to their unpurified counterparts, it was not eliminated. Further repurification attempts were not successful in eliminating completely the incorporation of [^{3}H]AdoMet in reactions without added GTP.

The possibility exists that GTP is endogenously present within purified virions. We have occasionally noticed a higher content of 2′-*O*-methylated bases than ^{7m}G in capped structures labeled in vitro (unpublished data). Since the latter methylation occurs prior to ribose methylation (17), it suggests that unlabeled AdoMet is endogenously present within purified vaccinia virions. Attempts at eliminating putative endogenous GTP by preincubating the virus before labeling with [^{3}H]AdoMet have not been successful, and the resolution of this problem requires further study.

At this point, we considered that the methylated low-molecular-weight RNA labeled in the absence of a full complement of the ribonucleoside triphosphates might be a useful device for measuring the number of promoters present on the vaccinia virus genome, and hence might allow us to distinguish a mono- from polycistronic mode of RNA synthesis by vaccinia virus. In addition, as will be discussed later, we considered that these methylated low-molecular-weight RNAs might be involved in mRNA biogenesis.

The methylated low-molecular-weight RNAs are polyadenylated. One of the implications suggested by the absolute requirement for ATP along with two or more other triphosphates for the methylation observed in vitro was that the methylated low-molecular-weight RNA molecules might also be polyadenylated. To test this, [^{3}H]AdoMet-labeled low-molecular-weight RNAs were synthesized in in vitro reactions by vaccinia virus in the presence of ATP, CTP, and UTP, or ATP, GTP, and CTP, or ATP, GTP, and UTP, or with all four ribonucleoside triphosphates in reactions containing 20 μg of Actino D per ml to inhibit functional mRNA synthesis. The extent of polyadenylation of the above methyl-labeled low-molecular-weight RNAs was then compared to the poly(A) content of vaccinia RNA synthesized under standard conditions with all four ribonucleoside triphosphates. As is demonstrated in Table 3, the methylated low-molecular-weight RNAs were polyadenylated. The extent of binding to poly(U) filters was as great as RNA and in most instances more efficient. Additional evidence for the presence of poly(A) at the 3′ termini of these low-molecular-weight RNAs was obtained by labeling the 3′ termini using cytidine 3′,5′-bisphosphate, [5′-^{32}P], and T_4 RNA ligase. The labeled material was shown to be resistant to digestion with pancreatic and T_1 RNases in 0.3 M NaCl, indicative of poly(A). Analysis of the

size of the labeled poly(A) tail on denaturing gels indicated a heterogeneous population from about 20 to 100 bases depending on the class of PALs used to derive the 3'-terminal poly(A). In each case the size of the labeled product was reduced approximately 30 to 60% after RNase digestion, indicating that the resistant moiety is attached to an RNase-sensitive region and that little free poly(A) is synthesized under these conditions (data not presented). The extent of polyadenylation of the low-molecular-weight RNA, however, is dependent on the concentration of ATP present in the in vitro reaction, as has been shown for the polyadenylation of viral RNA (26) (data not presented). It is with reference to the methylation and polyadenylation of these low-molecular-weight RNA molecules that we have elected to call them polyadenylated leaders or PALs.

Methyl group analysis of the low-molecular-weight RNAs synthesized by vaccinia virus in vitro. Methyl group constituents of the PALs were analyzed by standard enzyme digestions and subsequent resolution of the digestion products by paper chromatography. More than 95% of the [^{3}H]AdoMet radioactivity assayed was detected in the methylated cap structures (Table 4; percent distribution is noted in parentheses). Points to be noted from Table 4 are as follows. (i) Both types of cap structures, ^{m}GpppGm and ^{m}GpppAm, were detected in all types of PALs and RNA. (ii) Unlike RNA, which contains a significant number (approximately 50%) of caps methylated only at the ^{7m}G, all four PALs contained predominantly caps that are methylated at both the ^{7m}G and 2'*O* position of the penultimate base (83 to 95%). (iii) Only 5% of the cap structures from the ACU PAL are of the type GpppG. It has been noted previously (19, 22) that the ratio of GpppG to GpppA caps differs depending on either the concentration of virus or ATP. It is interesting (Table 4) that these ratios might reflect the transcription of certain classes of promoters as detected by the ratios of cap structure in PALs. It should also be noted from Table 4 that the ACU PAL with its predominant GpppA type cap (94%) is consistent with a very low, yet detectable level of GTP contamination. This might evidence itself, as here, in sufficient GTP concentrations to form capped structures but too low a concentration to significantly result in G-initiated RNAs. Cap analysis of the poly(A)$^-$ components of the PALs revealed distributions similar to those of the poly(A)$^+$ material (data not presented).

TABLE 3. *Poly(U) filter binding of [^{3}H]AdoMet-labeled RNA and PALs*[a]

RNA type	Acid-precipitable cpm	Poly(U)-binding cpm	% Binding to poly(U) filters
RNA	29,651	24,366	82
ACU PAL	13,600	13,027	96
AGC PAL	4,659	3,853	83
AGU PAL	7,904	7,107	90
ACGU + Actino D PAL	18,384	16,010	87

[a] [^{3}H]AdoMet-labeled RNA and PALs were either precipitated with trichloroacetic acid and collected on Whatman GF/C filters or diluted into 0.05 M Tris-hydrochloride (pH 7.4), 0.1% SDS, 0.4 M NaCl, and 1 mM trisodium EDTA and filtered through poly(U)-containing GF/C filters. The latter samples were also precipitated with trichloroacetic acid after appropriate washings. cpm, Counts per minute.

The methylated cap structures were further analyzed for the methylated nucleoside constituents by further enzymatic digestions and analysis by paper chromatography. Again, more than 95% of the radioactivity assayed was recovered in the methylated nucleosides (the distribution of the recovered radioactivity is noted in parentheses; Table 5). As noted from Table 5, the distribution of the methylated nucleosides is consistent with the distribution of caps in Table 4. Analysis of the methylated structures found in the PALs reveals constituents identical to those found in vaccinia virus mRNA (35).

Size of PALs on denaturing polyacrylamide gels. We have analyzed the size of both [^{3}H]AdoMet- and internally labeled PALs on denaturing polyacrylamide gels and report our results below. Figure 2 shows the distribution on 12% polyacrylamide gels of poly(A)$^-$ AGU (lane A) or poly(A)$^-$ ACG (lane C) PALs labeled with [α-^{32}P]UTP or [α-^{32}P]CTP, respectively. The discrete banding patterns should be noted. The corresponding poly(A)$^+$ PALs of AGU (lane B) and ACG (lane D) indicate few discrete bands, and a somewhat overexposed radioautogram is shown to point out the overlap of size contributed to the PALs by the poly(A) moiety. Approximately 80 to 90% of the poly(A)$^-$ AGU or ACG PALs fall within a range of 25 to 90 bases. Figure 3 shows the size distribution of [α-^{32}P]UTP-labeled poly(A)$^-$ ACU PALs (lane A) or poly(A)$^-$ Actino D PALs (lane E). The corresponding poly(A)$^+$ PALs are in lanes B and F, respectively. The size distributions of [α-^{32}P]CTP-labeled poly(A)$^-$ ACU PALs (lane C) or poly(A)$^-$ Actino D PALs (lane G) and their corresponding poly(A)$^+$ PALs (lanes D and H, respectively) are shown. To be noted from the radioautogram of this 3.5% polyacrylamide slab gel is the broad distribution of sizes of the

TABLE 4. *Analysis of methylated cap structures in vaccinia virus RNA and PALs synthesized in vitro*[a]

RNA type	[^{3}H]AdoMet cpm (%) in isolated cap structure			
	^{m}GpppG	^{m}GpppGm	^{m}GpppA	^{m}GpppAm
ACG PAL	3,435 (11)	16,770 (53)	1,200 (4)	10,311 (33)
ACU PAL	106 (0)	1,591 (5)	1,308 (4)	27,378 (90)
AGU PAL	2,592 (9)	11,470 (41)	1,986 (7)	11,655 (42)
Actino D PAL	2,242 (9)	7,969 (30)	2,148 (8)	13,916 (53)
Poly(A)$^+$ RNA	8,303 (31)	5,386 (20)	5,604 (22)	7,358 (28)

[a] Purified RNAs were digested in 0.01 M sodium acetate (pH 6.0) with P_1 nuclease at 37°C for 2 h. The reactions were then made 0.05 M with Tris-hydrochloride (pH 8.5) and 5 mM with $MgCl_2$ and digested with bacterial alkaline phosphatase for an additional 2 h. Cap structures were isolated by ascending paper chromatography on Whatman 3MM paper using isobutyric acid–0.5 M ammonium hydroxide (5:3). Numbers in parentheses indicate percentage of counts per minute (cpm) found in the noted structure.

TABLE 5. *Analysis of methylated nucleosides derived from the cap structure of vaccinia virus RNA and PALs synthesized in vitro*[a]

RNA type	[^{3}H]AdoMet cpm (%) in methylated nucleosides		
	^{m}G	Gm	Am
ACG PAL	13,972 (55)	6,373 (25)	5,128 (20)
ACU PAL	8,161 (47)	567 (3)	8,562 (50)
AGU PAL	9,630 (57)	3,805 (22)	3,481 (21)
Actino D PAL	8,058 (56)	2,302 (16)	4,009 (28)
Poly(A)$^+$ RNA	11,449 (72)	2,109 (13)	2,324 (15)

[a] Methylated nucleosides were prepared from cap structures by digestion with 6 U each of nucleotide pyrophosphatase and bacterial alkaline phosphatase per ml at 37°C for 2 h. The nucleosides were separated on Whatman 3MM paper using ascending chromatography in 2-propanol–water–57% NH_4OH (7:2:1). Numbers in parentheses indicate percentage of counts per minute (cpm) found in the noted structure.

ACU and Actino D PALs. These PALs fall within a range of sizes from approximately 60 to several hundred bases. The ability to form sharp bands suggests that they represent discrete molecules. Again, few bands were observed with the corresponding poly(A)$^+$ PALs. This failure to see bands in the poly(A)$^+$ PALs is probably due to the heterogeneity of the size of the poly(A) tail. Nevertheless, it should be noted that contribution to size made by the poly(A) tail is not inordinate; the size of poly(A)$^+$ PALs falls well within the general size distribution of the poly(A)$^-$ PALs. Worthy of note are the apparent complexity differences observed in either set of poly(A)$^-$ ACU or Actino D PALs labeled with [α-^{32}P]UTP or [α-^{32}P]CTP. This discrepancy is apparently due to the relative abundance of U or C in the PAL. Careful examination of Fig. 3 indicates that a number of bands in the ACU PALs or Actino D PALs have similar electrophoretic mobility but different intensities depending on whether the label comes from [α-^{32}P]UTP or [α-^{32}P]CTP. As will be noted below, such differences are obviated by labeling with [^{3}H]AdoMet at the 5′-terminal position.

The apparent discrepancy in the number of PALs resolved by gel analysis of internally labeled molecules might at first be considered to be an artifact of the system. However, when one analyzes the distribution of PALs on gels after labeling only at the 5′ end with [^{3}H]AdoMet, clearly reproducible patterns of defined complexity are seen. The patterns obtained with ^{3}H-labeled AGU poly(A)$^-$ PALs (Fig. 4) and ^{3}H-labeled ACG poly(A)$^-$ PALs (Fig. 5) are different from those of the internally labeled PALs (Fig. 2). The distribution observed here with terminally labeled PALs is quite reproducible from preparation to preparation. Analysis on similar gels of [^{3}H]AdoMet-labeled poly(A)$^+$ ACG or AGU PALs showed few discrete bands and a spectrum of electrophoretic mobility residing within the migration distances of the poly(A)$^-$ PALs (data not presented). The distribution of the ^{3}H-terminally labeled poly(A)$^-$ PALs of ACU and Actino D, the more complex PALs, is shown in Fig. 6. The fluorogram shows a high degree of reproducibility of ^{3}H-terminally labeled ACU and Actino D poly(A)$^-$ PALs. This reproducible pattern was obtained from PALs made with three different purified virus preparations. Identical patterns are also obtained using different batches of repurified triphosphates.

Hybridization of [^{3}H]AdoMet-labeled polyadenylated leaders to restriction fragments of vaccinia virus DNA. ^{3}H-methylated poly(A)$^+$ PALs were hybridized to *Hpa*II restriction fragments of vaccinia virus DNA immobilized to nitrocellulose filters. Each of the four sets of PALs has a defined hybridization pattern (Fig. 7). The greatest complexity when compared to [^{3}H]methyl-labeled RNA (lane I) was shown by Actino D (lane G) and ACU PALs (lane A), consistent with the complexity observed on gels. Next in complexity was the ACG PAL (lane C), followed by the AUG PAL (lane E). The latter shows hybridization to only a few

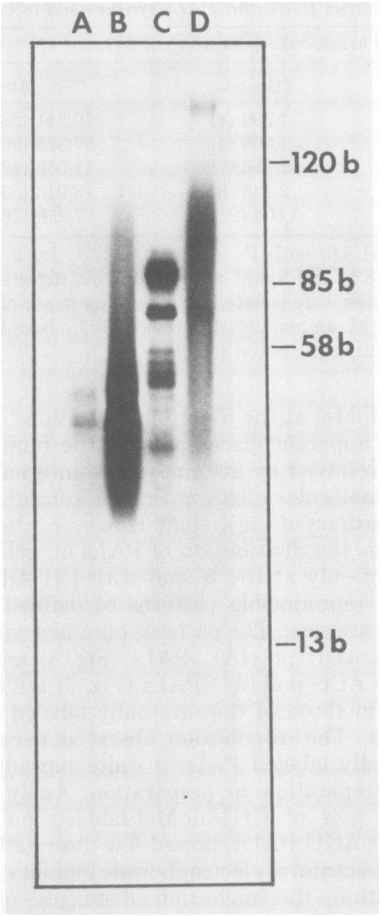

FIG. 2. *Analysis of AGU and ACG PAL by polyacrylamide gel electrophoresis. Poly(A)$^-$ AGU (lane A) or poly(A)$^-$ ACG (lane C) PALs labeled with [α-^{32}P]UTP or [α-^{32}P]CTP, respectively, and their corresponding poly(A)$^+$ components (lanes B and D) were analyzed by electrophoresis on 12% polyacrylamide-7 M urea gels as described in the text. The size of the PALs determined from the migration of 4S and 5S RNA, as well as the bromophenol blue and xylene cyanol$_{FF}$ markers, are noted on the side of the radioautogram.*

restriction fragments. This may be related to the size of the AUG PAL, since this gives the smallest size range on gels. Other explanations, however, are not excluded.

If the hybridization pattern of the PALs represents a measurement of promoters, then there are a very large number of promoters scattered throughout the vaccinia virus genome. If the PALs do in fact hybridize only to the promoters, then one could expect a high degree of resistance to digestion with pancreatic RNase in high salt when the PAL is in hybrid form. If, on the other hand, the hybridization pattern of the PALs represents both hybridization to primary sites of transcription and, in addition, hybridization to secondary sites on the DNA with sufficient complementarity to allow hybridization, then one might expect that a degree of sensitivity to pancreatic RNase digestion of the PALs might be

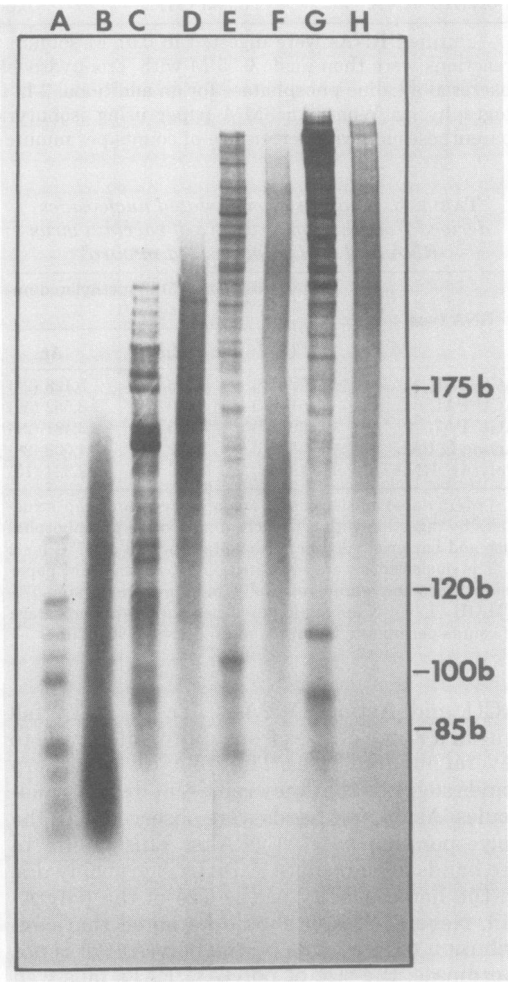

FIG. 3. *Electrophoretic analysis of ACU and Actino D PALs on 3.5% polyacrylamide-7 M urea gels. [α-^{32}P]UTP-labeled poly(A)$^-$ ACU PALs (lane A) or poly(A)$^-$ Actino D PALs (lane E) and their corresponding poly(A)$^+$ PALs (lanes B and F, respectively) were analyzed by electrophoresis on 3.5% polyacrylamide gels containing 7 M urea. [α-^{32}P]CTP-labeled poly(A)$^-$ ACU PALs (lane C), poly(A)$^-$ Actino D PALs (lane G), and their corresponding poly(A)$^+$ PALs (lanes D and H, respectively) are also shown. The relative size of the PALs determined from the migration of 4S and 5S RNA as well as the bromophenol blue and xylene cyanol$_{FF}$ markers are noted on the side of the radioautogram.*

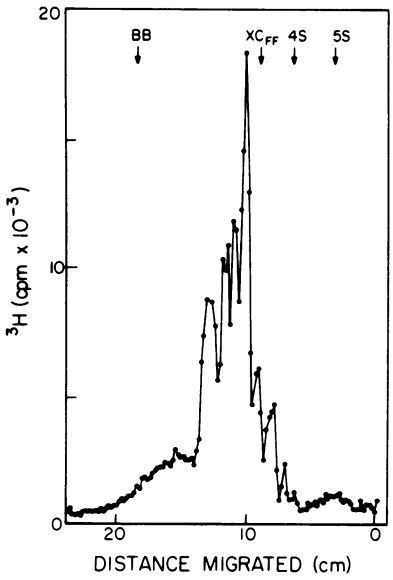

FIG. 4. *Electrophoretic analysis of [^{3}H]AdoMet-labeled poly(A)$^-$ AGU PALs. Poly(A)$^-$ AGU PALs labeled at the 5'-terminus with [^{3}H]AdoMet were analyzed on cylindrical 12% polyacrylamide-7 M urea gels. The gel was sliced into 2-mm sections and hydrolyzed with H_2O_2, and radioactivity was counted. The mobility of 4S and 5S RNA and dye markers bromophenol blue (BB) and xylene cyanol$_{FF}$ (XC_{FF}) markers obtained from parallel gels are also shown.*

observed at these secondary hybridization sites. Such has been the observation with adenovirus, where the 5'-terminal portion of the RNA is sensitive to pancreatic RNase digestion by virtue of its being spliced to RNAs that hybridize distal to the site of transcription of the 5'-terminal piece (11, 15). To test these alternatives, ^{3}H-terminally labeled PALs were hybridized to vaccinia virus DNA restriction fragments fixed to nitrocellulose filters, and their sensitivity to digestion with pancreatic RNase was measured. Considerable sensitivity of the ^{3}H-labeled 5' end of the ACU (Fig. 7, lane B) and Actino D (lane H) PALs was observed upon digestion with pancreatic RNase. This sensitivity was also evident with [^{3}H]methyl-labeled RNA (lane J), but not as significantly with RNA labeled internally with [^{3}H]UTP (lane L).

Base composition of the PALs. It was of interest to measure more accurately the level of contamination of the missing ribonucleoside triphosphate in the PALs synthesized in the presence of ATP and two other ribonucleoside triphosphates. To do this we synthesized cDNA from each of the poly(A)$^+$ PALs as well as RNA in separate reactions, each with a specific labeled [^{3}H]dNTP. The base composition of the PALs was then deduced from the base composition of the cDNA synthesized. The base composition of the PALs derived from the reverse transcription analysis is given in Table 6. Each of the four PALs exhibited a relatively high mole percentage of adenosine, as expected. The measurable level of "contamination" of the missing ribonucleoside triphosphate was 0.6, 2.9, and 2.0% in the ACG, AGU, and ACU PALs, respectively. Nevertheless, it is clear that this level of extraneous triphosphate is sufficiently low to generate the synthesis of discrete molecules as measured by a number of different criteria. That the composition of the PALs represents an accurate measurement using the cDNA analysis is supported by reverse transcription of vaccinia RNA. As is demonstrated in Table 6, the base composition of RNA is approximately 36% guanine plus cytosine, as deduced from cDNA synthesis, equivalent to the guanine plus cytosine content of vaccinia DNA. Further, analysis of the cDNA made to the PALs on gels shows a distribution in size of the cDNA corresponding very well with the PALs used as template, and the cDNA, when hybridized to DNA restriction fragments, gives patterns of hybridization identical to the respective PALs or RNA used as template (data

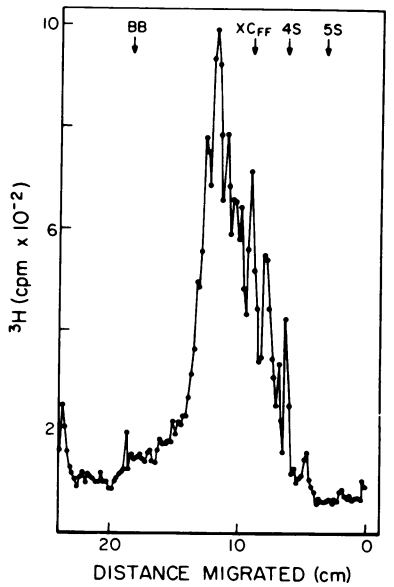

FIG. 5. *Electrophoretic analysis of [^{3}H]AdoMet-labeled poly(A)$^-$ ACG PALs. Poly(A)$^-$ ACG PALs labeled at the 5'-terminus with [^{3}H]AdoMet were analyzed on cylindrical 12% polyacrylamide-7 M urea gels. The gel slices (2 mm) were hydrolyzed with H_2O_2, and radioactivity was counted. The mobilities of 4S and 5S RNA and the dye markers bromophenol blue (BB) and xylene cyanol$_{FF}$ (XC_{FF}) are also shown.*

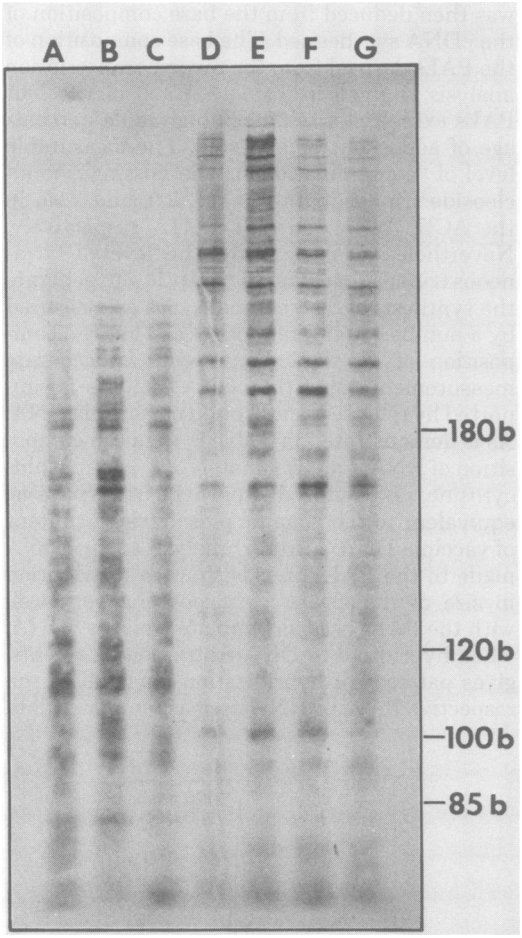

FIG. 6. *Fluorogram of [^{3}H]AdoMet-labeled ACU and Actino D poly(A)$^-$ PALs analyzed on polyacrylamide gels. Four different preparations of poly(A)$^-$ Actino D PALs (lanes D-G) and three different preparations of poly(A)$^-$ ACU PALs (lanes A-C) were analyzed on 3.5% polyacrylamide-7 M urea gels. The gels were fluorographed as described by Bonner and Laskey (4). The relative size of the PALs is noted from the electrophoretic mobility of 4S and 5S RNA and the bromophenol blue and xylene cyanol$_{FF}$ markers.*

not presented). We are aware that the contribution of the poly(A) region in the PALs will contribute to the relative mole percentage of adenosine in the PALs. Therefore, additional information on the base composition of the PALs and their non-poly(A) moiety was obtained by performing a nearest-neighbor analysis of poly(A)$^+$ and poly(A)$^-$ molecules labeled with [α-^{32}P]ATP. The results (Table 7) are consistent with the data obtained by reverse transcription. It should be noted (Table 7) that the "missing" triphosphate is found in the non-poly(A) moiety at levels comparable with the added triphosphates.

DISCUSSION

We have described the synthesis and partial characterization of low-molecular-weight RNA molecules made by vaccinia virus in vitro. Although a number of small, nonribosomal, nontransfer RNAs have been described in the nuclei and cytoplasm of a variety of noninfected or virus-infected eucaryotic cells (2, 18, 28, 31, 36, 37), virtually nothing is known about their biological function. The low-molecular-weight RNA molecules synthesized by vaccinia virus in vitro and described here are unique in that they are modified at both termini, being capped and methylated at the 5′ terminus and polyadenylated at the 3′ terminus.

What is the significance of these small RNA molecules, particularly with reference to the vaccinia virus system? Are they artifacts of the conditions of synthesis used? Similar type molecules are observed during in vitro RNA synthesis both at very early times and at late times after ATP has become depleted. We have not looked for the synthesis of these PALs in vivo, but it is noteworthy to recall the data of Rosemond-Hornbeak and Moss (29), who showed that in the presence of Actino D vaccinia virus synthesized in vivo small poly(A)-rich RNA molecules and implicated these molecules in the rapid shut-off of host macromolecular synthesis. It would not be at all surprising if these molecules were also capped and methylated. We have also demonstrated that the PALs described here inhibit quite dramatically the translation of vaccinia virus mRNA in vitro while not being themselves translated (6; unpublished data). The chemical modifications present at both termini make these ideal candidates for involvement in the rapid shut-off of host macromolecular synthesis upon infection, by virtue of a rapid displacement of host mRNA from the ribosomes, or they may facilitate the preferential translation of viral mRNA by some as yet undefined mode.

It is clear from our data that the small RNA molecules described here are not simply poly(A). They are not synthesized in the presence of ATP or ATP and GTP. Further, poly(A) synthesis by vaccinia does not require that the virus be exposed to —SH reagents. RNA and PAL syntheses, on the other hand, are both absolutely dependent upon the presence of —SH groups during synthesis. In addition, whereas poly(A) shows no lag in synthesis, the synthesis of PALs as with RNA shows a detectable lag of several minutes (unpublished data).

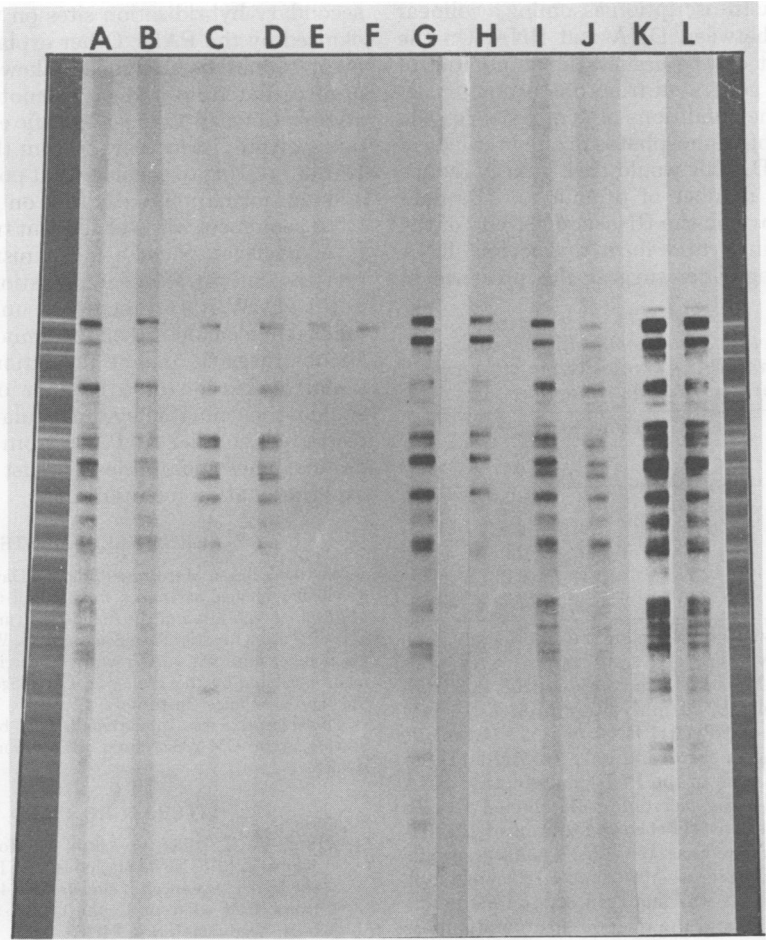

FIG. 7. *Hybridization of PALs and RNA to HpaII restriction fragments of vaccinia DNA. PALs were hybridized at a constant radioactivity of approximately 7 × 10⁵ cpm/ml, and RNAs were hybridized at 10⁶ cpm/ml. The HpaII restriction pattern obtained with vaccinia DNA is shown in the extreme lanes of the photograph. The hybridization pattern of the PALs and RNA is as follows: [³H]AdoMet-labeled ACU PALs (lanes A and B); ACG PALs (lanes C and D); AUG PALs (lanes E and F); Actino D PALs (lanes G and H); and RNA (lanes I and J). RNA labeled internally with [³H]UTP is shown in lanes K and L. Lanes B, D, F, H, J, and L were digested with pancreatic RNase. The exposure time was 25 days. HpaII cleaves vaccinia DNA into approximately 60 fragments ranging in size from ~6 to 0.3 kilobase pairs.*

Is the point of poly(A) addition a specific sequence? We cannot speak to the question of specificity of polyadenylation from the data presented here. However, one might argue that poly(A) addition occurs here only because of the "abortive" conditions of synthesis. It should be pointed out that analysis of T_1 digestion indicates that the average 3'-terminal sequences of vaccinia virus mRNA are —$G(C_2U_4A)\ A_{(100)}$ (21). Furthermore, the poly(A) polymerase associated with the virus, isolated from infected cells or purified from vaccinia virions (7, 8, 19), was not able to efficiently polyadenylate large RNA molecules but very readily polyadenylated short oligonucleotides with little specificity regarding base composition. It is interesting, again, that analyses of the poly(A)⁻ PALs on gels show discrete-sized molecules. Although we do not exclude that these poly(A)⁻ PALs contain a number of adenosine residues at the 3' terminus, the number is sufficiently small so as not to bind to poly(U)-Sepharose and not to represent an overlap of A residues which might be expected to eliminate the banding characteristics of the poly(A)⁻ PALs.

It can be argued from the data that there is a very large number of promoters on the vaccinia genome, a number large enough to account for

monocistronic transcription assuming a colinear relationship between DNA and RNA. On the other hand, it can be argued that a number of PALs can be generated from one promoter depending on the conditions of synthesis, i.e., the combination of triphosphates or the presence of actinomycin D. This would then greatly reduce the apparent number of promoters. The data presented above on the RNase sensitivity of the PALs when in hybrid form to vaccinia DNA restriction fragments suggest the presence of secondary hybridization sites on the DNA recognized by the PALs. Other explanations, however, cannot be excluded. Likewise, it can be argued that from a given promoter locus polyadenylation can still be a specific event throughout a given distance away from that promoter. It is interesting to consider that polyadenylation and cap formation occur first on small defined RNA sequences with subsequent transfer to mature messages. Such a mechanism could conserve essentially all the informational sequences in the HMW RNA precursors, unlike the splicing mechanism described for simian virus 40 and adenoviruses (1, 3). The regulation of the functional expression of a number of viral genes could be coordinated by the regulation of a more restricted number of PALs from defined promoters. These points clearly must await further experimentation for resolution.

TABLE 6. *Relative base composition of vaccinia virus RNA and PALs synthesized in vitro from analysis of reverse transcription products*[a]

RNA type	Relative moles percent			
	A	C	G	U
ACG PAL	94	3.4	2.0	0.6
AGU PAL	88	2.9	4.5	4.6
ACU PAL	90.6	5.0	2.0	2.4
Actino D PAL	74.7	10.4	6.2	8.7
RNA	40.7	17.1	18.7	23.5

[a] cDNA was synthesized using the (100-U/ml) avian myeloblastosis virus reverse transcriptase in reactions containing 50 mM Tris-hydrochloride (pH 8.4), 60 mM NaCl, 4 mM $MgCl_2$, 20 mM dithiothreitol, 0.6 mM dNTP's, 100 μg of poly(A)$^+$ RNA or PALs per ml, 20 μg of oligo(dT)$_{12-18}$ per ml, and 100 μg of Actino D per ml. cDNA from each of the PALs and poly(A)$^+$ RNA was synthesized using each of four ^{3}H-labeled dNTP's in separate reactions. The concentration of the ^{3}H-labeled dNTP was reduced to 0.1 mM, and the specific activities were kept between 862 and 1,129 cpm/pmol. The cDNA product was analyzed at six time points from 5 min to 1 h, and the relative incorporation was computed for the complementary bases in the vaccinia viral RNAs.

TABLE 7. *[α-^{32}P]ATP incorporation and nearest-neighbor analysis*[a]

RNA type	% ^{32}P associated with:			
	Ap	Gp	Cp	Up
Poly(A)$^+$				
ACG PAL	93	3	2	2
AGU PAL	94	2	1	3
ACU PAL	89	3	4	4
Actino D PAL	87	4	3	6
RNA	43	19	12	26
Poly(A)$^-$				
ACG PAL	40	17	20	23
AGU PAL	56	9	15	20
ACU PAL	32	20	16	32
Actino D PAL	33	18	18	31
RNA	31	21	16	32

[a] Nearest-neighbor analysis was performed as described by Volckaert and Fiers (34) using [α-^{32}P]ATP-labeled PALs.

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