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Cell-Free Translation of Purified Virion-Associated High-Molecular-Weight RNA Synthesized In Vitro by Vaccinia Virus

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Virion-associated high-molecular-weight (HMW) RNA synthesized in vitro by purified vaccinia virus particles has been translated in a wheat germ cell-free protein synthesizing system. Purified HMW RNA directs the synthesis of translation products which are identical to the translation products made in response to in vitro-synthesized, virion-released 8 to 12S mRNA. The translation of HMW RNA proceeds exclusively through a 5′-terminal cap-mediated initiation step. Furthermore, only one coding sequence is translated per HMW RNA molecule, and that sequence is probably located near the 5′ end of the molecule. These conclusions are based on the following results. (i) Sodium dodecyl sulfate—polyacrylamide gel electrophoresis patterns of translation products synthesized in response to HMW RNA and in response to 8 to 12S mRNA were qualitatively identical. (ii) On an equal weight basis, HMW RNA was 25 to 30% as active as 8 to 12S mRNA in stimulating in vitro protein synthesis. (iii) Unmethylated HMW RNA was translated at 10% the efficiency of the methylated form of this RNA. (iv) m^7pG inhibited the translation of fully methylated HMW RNA by 90%. (v) After the initiation step of translation was blocked by aurintricarboxylic acid, the rate with which amino acids were incorporated into individual polypeptides decreased in a similar manner for the translation of both HMW RNA and 8 to 12S mRNA. Virion-released 8 to 12S mRNA derived from virion-associated HMW RNA during a chase in the presence of ATP, GTP, and *S*-adenosylmethionine was also translated. At low RNA concentrations, the derived RNA appeared to stimulate amino acid incorporation more efficiently than the HMW RNA precursor. However, at higher concentrations of this RNA, protein synthesis was severely inhibited.

RNA synthesized and extruded by vaccinia virus particles in vitro has an average sedimentation value of between 8 and 12S (15, 35), contains a 7-methylguanosine cap at the 5′ terminus (36), and is polyadenylated at the 3′ end (16, 24). Under appropriate conditions, vaccinia virus particles preferentially synthesize a high-molecular-weight (HMW), 20 to 30S form of RNA (26, 29a). These large transcripts are found only in association with the virus cores and are not released from the virion as HMW RNA molecules (26, 27, 29a). HMW RNA is also capped at the 5′ end (25), but it is not extensively polyadenylated at the 3′ end (27, 29a). This virion-associated HMW RNA can be chased within the virion particle to RNA in the 8 to 12S range, some of which is released from the virus (27). This cleavage process is not dependent on continued RNA synthesis but does require ribonucleoside triphosphates and is prevented by the addition of ethidium bromide (27). Recently, an endoribonuclease which appears specific for the cleavage of the virion-associated HMW RNA has been solubilized from vaccinia virus particles (29). Furthermore, the transcriptional complexity of virion-released 8 to 12S mRNA and virion-associated HMW RNA is identical (28). These combined results suggest a precursor-product relationship between virion-associated HMW RNA and virion-released 8 to 12S mRNA.

Recently both we (7) and H. Pelham (30) described the effect of UV irradiation on the expression of vaccinia virus gene products synthesized in a cell-free system coupling transcription and translation. Although different translation systems were used in these two studies, similar results were obtained. These results in-

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dicated that each translationally functional mRNA species produced in vitro by vaccinia virus cores is synthesized from individual promoter sites. However, we could not rule out the possibility that the polypeptides expressed in the coupled system are the products of sequences found very near the 5′ end of several long transcriptional units, whereas other sequences that are expressed only after normal processing of these long transcripts are translationally silent in the in vitro translation system. To investigate this possibility and to further characterize the virion-associated HMW RNA, we have determined a number of parameters with respect to the translation of this RNA in a wheat germ cell-free protein-synthesizing system.

MATERIALS AND METHODS

Synthesis and purification of vaccinia RNA. Vaccinia virus (WR) was grown in and purified from HeLa cells as described by Joklik (13). RNA was synthesized in a standard in vitro RNA polymerase reaction consisting of 50 mM Tris-hydrochloride (pH 8.4), 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.05% Nonidet P-40, 2 mM each ATP, GTP, and CTP, and 0.2 mM [^{3}H]UTP, (0.025 Ci/mmol). Purified virus was added to a final concentration of 4 to 5 optical density units at 260 nm per ml. Incubation was at 37°C.

For the preparation of the methylated and unmethylated forms of virion-released 8 to 12S mRNA, the RNA polymerase reaction was incubated at 37°C for 30 min in the presence of either 10 μm of *S*-adenosylmethionine (AdoMet) or 100 μm of *S*-adenosylhomocysteine (AdoHcy). Virus was removed from the reaction mixture at the end of the incubation time by centrifugation at 15,000 rpm for 30 min. RNA was phenol extracted from the virus-free reaction mixture and purified on sodium dodecyl sulfate (SDS)—sucrose gradients as previously described (25, 27).

For the preparation of the methylated and unmethylated forms of HMW RNA, reaction mixtures containing either AdoMet or AdoHcy were preincubated for 20 min in the absence of UTP to reduce the ATP concentration (29a), and synthesis was then started by the addition of [^{3}H]UTP (0.025 Ci/mmol) to a final concentration of 0.2 mM. After an incubation period of 40 min, RNA was extracted from the virus particles as described previously (26, 27, 29a), and then purified by sedimentation through SDS—sucrose gradients. Both types of RNA were recovered from the gradients by ethanol precipitation. Three additional ethanol precipitations were performed to remove all traces of SDS before the addition of RNA to the cell-free translation system.

Cell-free translation. A cell-free protein-synthesizing system was prepared from Niblack brand wheat germ by the method of Roberts and Paterson (32), excluding the preincubation step. Reaction mixtures for cell-free translation contained wheat germ extract at a final concentration of 25 optical density units at 260 nm per ml; 20 mM *N*-2-hydroxyethyl piperazine-*N*′-ethanesulfuric acid (pH 7.5); 135 mM KOAc; 3.5 mM $Mg(OAc)_2$; 1.25 mM ATP; 0.25 mM GTP; 15 mM creatine phosphate (Sigma Chemical Co.); 75 μg of creatine phosphokinase (Sigma) per ml.; 3.6 mM dithiothreitol; 0.3 mM spermidine—hydrochloride; 50 μM each amino acid minus either leucine or methionine; and either 200 μCi of [^{3}H]leucine per ml (54.6 Ci/mmol; New England Nuclear Corp.) or 500 μCi of [^{35}S]methionine per ml (573 Ci/mmol; New England Nuclear). In some reactions, AdoHcy was included at a concentration of 0.3 mM to inhibit methyltransferase activity present in the wheat germ extract.

RNA samples were heated at 60°C for 1 min and subsequently quenched on ice just before addition to the translation system to reduce any RNA—RNA aggregation. Protein synthesis was monitored as described by Mans and Novelli (21).

Polyacrylamide gel electrophoresis. Samples from translation reaction mixtures were prepared and analyzed by electrophoresis on 15% polyacrylamide gels followed by autoradiography as described previously (7). Radioactive polypeptides were detected on some gels by the fluorographic method of Bonner and Laskey (4).

RESULTS

Stimulation of protein synthesis by virion-associated HMW RNA. Purified vaccinia virion-associated HMW RNA, synthesized under recently established conditions which permit the preferential synthesis of these large transcripts (29a), was tested for the ability to stimulate protein synthesis in a wheat germ cell-free translation system. The level of amino acid incorporation into protein which was directed by virion-released 8 to 12S mRNA or virion-associated HMW RNA is shown in Fig. 1A and B, respectively, as a function of RNA concentration. Notice that both RNA populations stimulate protein synthesis to the same extent at the concentration of RNA which is saturating. However, the concentration of RNA which gives this maximal stimulation is considerably lower for the 8 to 12S mRNA than for the HMW RNA. The concentration of 8 to 12S mRNA which gave maximal stimulation of protein synthesis was consistently found to be between three- and fourfold lower, on an equal weight basis, than the concentration of HMW RNA which gave maximal protein synthesis under identical conditions. In this regard, sedimentation analysis has shown that the HMW RNA is approximately three- to fourfold larger than 8 to 12S mRNA (25–27). Furthermore, a comparison of the incorporation of methyl groups at the 5′ end with the incorporation of UMP at internal sites of these RNAs indicated that, on an equal weight basis, 8 to 12S mRNA has three- to fourfold more capped 5′-terminal ends than HMW RNA

synthesized under identical conditions (25).

Analysis by SDS-gel electrophoresis of polypeptides synthesized in response to virion-associated HMW RNA and virion-released 8 to 12S mRNA revealed that these two populations of RNA directed the synthesis of similar translation products. (Compare lanes A and B in Fig. 2.) There are some observable differences in the relative synthesis of the larger (greater than 50,000 molecular weight) polypeptides when comparing the translation products coded for by HMW RNA with the translation products programmed by 8 to 12S mRNA; the larger polypeptides are more prominent in translation reactions programmed by HMW RNA. Longer exposure of the 8 to 12S mRNA translation products does reveal, however, that the synthesis of polypeptides as large as 90,000 daltons is directed by this population of RNA. These quantitative differences are probably due to the sizing of these RNAs on sucrose gradients as part of the purification scheme. It is important to note that the synthesis of large polyproteins did not occur during the translation of the HMW RNA. This result suggests that normal termination signals are recognized by ribosomes translating the large transcripts.

We recently described a coupled transcription-translation system in which we determined

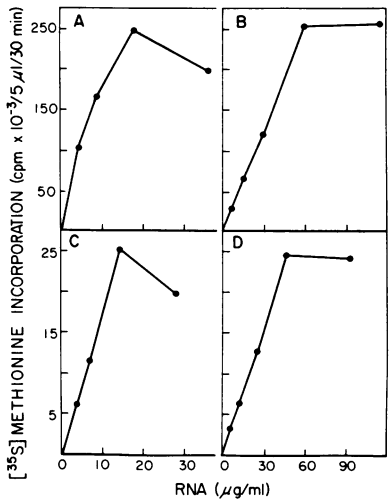

FIG. 1. *Stimulation of polypeptide synthesis in a wheat germ cell-free translation system in response to the methylated and unmethylated forms of HMW RNA and 8 to 12S mRNA. The methylated and unmethylated forms of HMW RNA and 8 to 12S mRNA were synthesized and purified as described in the text. Translation was in the presence of 0.3 mM AdoHcy for 30 min at 22°C. The level of amino acid incorporation directed by each type of RNA is presented as a function of RNA concentration. (A) Methylated 8 to 12S mRNA; (B) methylated HMW RNA; (C) unmethylated 8 to 12S mRNA; (D) unmethylated HMW RNA.*

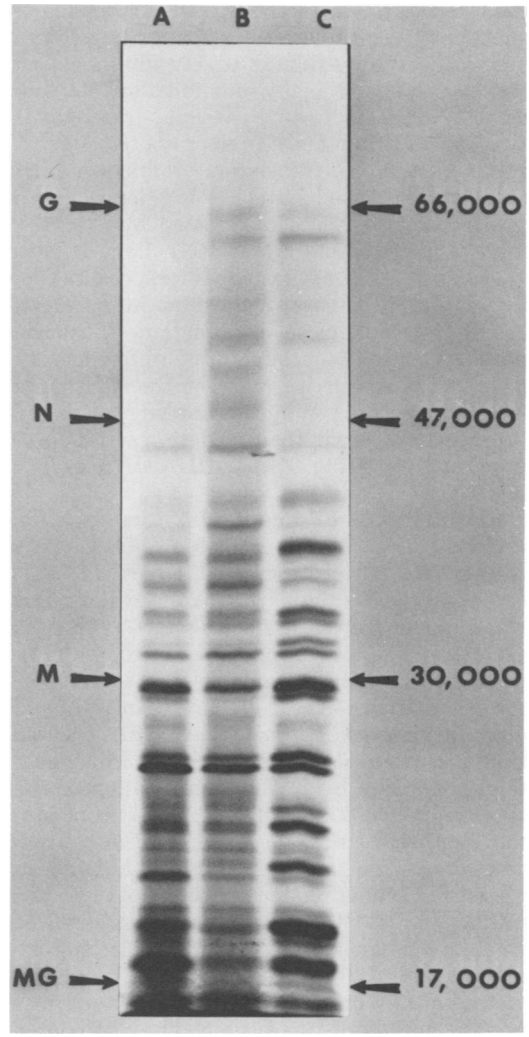

FIG. 2. *Autoradiograph of an SDS-polyacrylamide gel after electrophoresis of translation products synthesized in a coupled transcription-translation system or in response to exogenous 8 to 12S mRNA or HMW RNA. [35S]methionine-labeled translation products synthesized in the coupled system (lane C) as previously described (3) or synthesized under standard translation conditions in response to purified 8 to 12S mRNA (lane A) or purified HMW RNA (lane B) were analyzed by SDS-gel electrophoresis. Each well received 500,000 acid-precipitable cpm. The migration positions of marker proteins of known molecular weight are indicated on the left margin. The exposure time was 4 days.*

the effect of UV irradiation on the expression of vaccinia gene products in a wheat germ cell-free system (7). The results of that study suggested that each translationally functional mRNA species produced in vitro by vaccinia virus cores is synthesized from individual promoter sites. A comparison of the translation products synthesized in the coupled system (Fig. 2, lane C) with the translation products directed by 8 to 12S mRNA (lane A) and HMW RNA (lane B) also showed some quantitative differences in the translation products synthesized in all three cases. However, close examination of the gel patterns revealed no qualitative differences, at this level of sensitivity, between the translation products synthesized in the coupled system and those synthesized in the translation system programmed by either HMW RNA or 8 to 12S mRNA.

Requirement of a capped 5′ terminus for efficient translation of HMW RNA. It is known that virion-associated HMW RNA is capped at the 5′-terminal end (25). To determine whether the translation of this RNA proceeds exclusively via a 5′-terminal cap-mediated initiation step, the following experiments were performed. Virion-released 8 to 12S mRNA and virion-associated HMW RNA were purified from in vitro transcription reactions which contained AdoHcy to prevent methylation of the transcription products. The stimulation of protein synthesis with respect to the RNA concentration was then determined for the fully methylated and unmethylated forms of both 8 to 12S mRNA and HMW RNA. In this experiment, the wheat germ translation system was also supplemented with 0.3 mM AdoHcy to prevent methylation of any unmethylated RNA by enzymes present in the cell-free system. The methylated and unmethylated forms of each type of RNA exhibited maximal stimulation of protein synthesis at the same RNA concentration, i.e., 12 to 17 μg of both methylated and unmethylated 8 to 12S mRNA per ml (Fig. 1A and C) and 55 to 65 μg of both forms of the HMW RNA per ml (Fig. 1B and D). However, the unmethylated form of both 8 to 12S mRNA and HMW RNA stimulated protein synthesis only 10% as efficiently as the methylated forms of these RNAs. The requirement of a 5′-terminal cap for the efficient translation of vaccinia 8 to 12S mRNA has previously been reported (34). The data presented in Fig. 1 suggest that the efficient translation of HMW RNA also depends on the presence of a 5′-terminal cap.

The translation of mRNA's which normally require a cap for efficient translation is inhibited by the addition of m^7pG (11). The translation of mRNA's which do not require the cap for efficient translation, e.g., EMC RNA (33) and, presumably, the translation of RNA sequences which is initiated at internal initiation sites, is not affected by the addition of m^7pG at a concentration which inhibits the translation of capped mRNA (14). The effect of m^7pG on the translation of the methylated and unmethylated forms of both 8 to 12S and HMW RNA was tested (Table 1). The effect of m^7pG on the translation of both the 8 to 12S mRNA and the HMW RNA populations was identical. The translation of the methylated forms of both RNAs was inhibited by 90% when 1 mM m^7pG was added to the cell-free system, whereas this concentration of m^7pG had no effect on the translation of the unmethylated forms of these two RNAs. In determining the effect of m^7pG on the translation of unmethylated RNA, one must be cognizant of the observation that the addition of any RNA, e.g., rRNA (Table 1), results in a stimulation of endogenous wheat germ protein synthesis in translation systems which are not preincubated. This endogenous protein synthesis is completely inhibited by the addition of

TABLE 1. *Effect of m^7pG on the translation of the methylated and unmethylated forms of HMW RNA and 8 to 12S mRNA*

Reaction mixture[a]	[^{35}S]methionine incorporation after (min):[b]				
	0	10	20	30	60
−RNA					
$-m^7pG$	8.7	11.4	13.9	16.1	19.5
$+m^7pG$	8.5	8.1	8.5	8.3	7.1
rRNA (10 μg/ml)					
$-m^7pG$	7.9	15.7	22.1	25.3	33.5
$+m^7pG$	7.6	9.2	9.2	10.0	9.1
Methylated 8 to 12S mRNA (10 μg/ml)					
$-m^7pG$		70.4	163.9	230.6	276.8
$+m^7pG$		6.3	12.3	16.7	22.7
Unmethylated 8 to 12S mRNA (10 μg/ml)					
$-m^7pG$		6.4	13.2	18.4	21.5
$+m^7pG$		6.7	14.5	20.4	22.9
Methylated HMW RNA (40 μg/ml)					
$-m^7pG$		89.7	186.9	247.0	280.0
$+m^7pG$		7.3	15.1	20.6	23.3
Unmethylated HMW RNA (40 μg/ml)					
$-m^7pG$		3.7	9.5	13.1	19.4
$+m^7pG$		4.4	10.9	19.2	22.9

[a] The methylated and unmethylated forms of HMW RNA and 8 to 12S mRNA were translated under standard conditions in the presence or absence of 1 mM m^7pG. AdoHcy was present at a concentration of 0.3 mM in all reaction mixtures to prevent methylation of unmethylated RNA by the wheat germ methyltransferase.

[b] Results are presented as counts per minute × 10^{-3} of [^{35}S]methionine incorporation into hot acid-precipitable radioactivity per 5 μl of reaction mixture.

m^7pG. Thus, the stimulation of endogenous protein synthesis by the addition of unmethylated RNA and the inhibition of that endogenous protein synthesis by m^7pG must be taken into account when calculating the effect of m^7pG on the actual translation of unmethylated RNA. The results presented here with respect to the relative translation of the methylated and unmethylated forms of the 8 to 12S mRNA and the HMW RNA, as well as the effect of m^7pG on the translation of these RNAs, are in total agreement with previously published results for 8 to 12S mRNA (34) and strongly suggest that the translation of HMW RNA also proceeds exclusively through a 5'-terminal cap-mediated initiation step.

As a further test of this conclusion, the translation products directed by both methylated and unmethylated HMW RNA and 8 to 12S mRNA in the presence and absence of m^7pG were compared by SDS-gel electrophoresis. If any of the HMW RNA sequences are translated from an internal initiation site, then the corresponding polypeptide would be very prominent in the gel analysis of translation products directed by the unmethylated form of the HMW RNA. Also, the synthesis of these polypeptides would not be inhibited after the addition of m^7pG. As shown in Fig. 3, the gel profiles of the translation products of 8 to 12S mRNA, which are synthesized only from 5'-terminal initiation sites, are similar to the profiles of HMW RNA translation products synthesized under each set of conditions. Thus, we can conclude that the translation of HMW RNA proceeds through a 5'-terminal cap-mediated initiation step and can rule out the possibility that there is a significant level of initiation of HMW RNA translation at internal initiation sites.

Number and probable location of translated sequences on the HMW RNA molecule. Although the data presented in Fig. 1 and 2 and in Table 1 rule out the possibility that the translation of HMW RNA starts at internal initiation sites, it is possible that multiple sequences well downstream from the 5' end of the HMW RNA are translated after translation is initiated at the 5' end of the molecule, as has been determined for the translation of some coliphage mRNA (18). To investigate this possibility, the following experiment was performed. Translation of 8 to 12S mRNA and HMW RNA was allowed to initiate and proceed for 4 min in the absence of isotope, at which time the inhibitor of polypeptide chain initiation, aurintricarboxylic acid (ATA), was added to a final concentration of 75 μM. The ATA-inhibited reaction mixtures were then supplemented with the labeled amino acid either at the time of ATA addition or at 1-min intervals after the addition of ATA, and elongation was allowed to proceed for a total of 30 min. This experiment measures the time it takes for ribosomes to finish the translation of the mRNA molecules on which they had initiated before ATA addition, i.e., the runoff time. A runoff time for HMW RNA lasting three to four times longer than the runoff time for 8 to 12S mRNA would suggest that sequences all along the HMW RNA molecule are being translated after initiation at the 5' end. Identical runoff times for HMW RNA and 8 to 12S mRNA would suggest that only one coding sequence per HMW RNA was translated. The runoff kinetics were identical for both 8 to 12S mRNA and HMW RNA (Fig. 4).

As a final test, the translation products labeled during the runoff of both 8 to 12S mRNA and HMW RNA were analyzed by SDS-gel electrophoresis. The time required for runoff, as determined by the decrease in the level of radioactivity incorporated into polypeptides with time after ATA addition, increases progressively with increasing molecular weight of the polypeptides coded for by the 8 to 12S mRNA (Fig. 5A). From these measurements, a ribosome transit time of approximately 40 codons per min was calculated. A comparison of the gel patterns in Fig. 5A and B reveals that the individual polypeptides coded for by both the 8 to 12S mRNA and the HMW RNA have identical runoff kinetics.

With a ribosome transit time of 40 codons per min (calculated from data in Fig. 5), a ribosome which initiates at zero time can proceed only about 160 codons from the ribosome binding site by 4 min, the time of ATA addition; 160 codons represents about 70% of the coding region of an mRNA coding for a polypeptide of molecular weight 30,000. Since the initiation of HMW RNA translation, like the initiation of 8 to 12S mRNA translation, proceeds through a 5'-terminal cap-mediated step, the similar runoff kinetics for polypeptides coded for by HMW RNA and 8 to 12S mRNA suggests that the one sequence translated from each HMW RNA molecule is located near the 5' end of that molecule. However, it is conceivable, though unlikely, that the secondary structure of the large transcripts is such that the 5' terminus is in an unusually close functional association with the initiator codon for a coding sequence near the 3' end of the HMW RNA.

In preliminary experiments we determined the stability of both the HMW RNA and 8 to 12S mRNA in the wheat germ system by sucrose gradient analysis of the RNA's recovered from the translation system with increasing time of

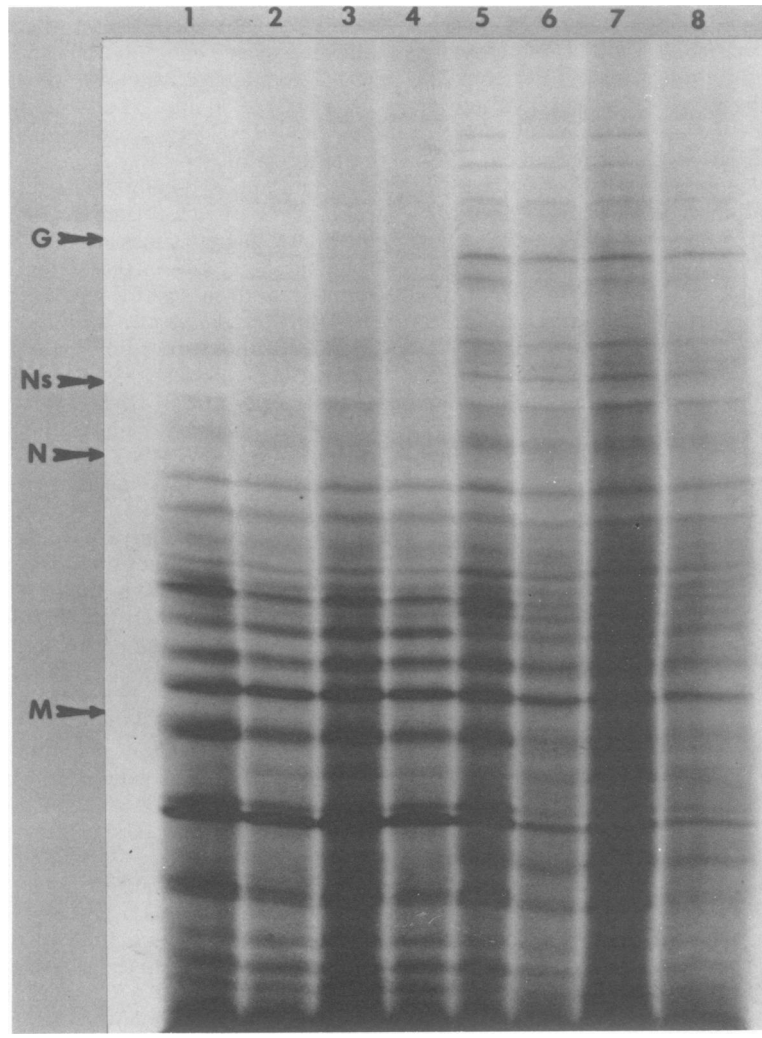

FIG. 3. *Autoradiograph of a SDS-polyacrylamide gel after electrophoresis of translation products synthesized in response to methylated and unmethylated HMW RNA and 8 to 12S mRNA in the presence or absence of 1 mM m^7pG. Each well received 120,000 acid-precipitable cpm. Samples were normalized with respect to protein concentration by adding appropriate amounts of non-radioactive wheat germ extract. (Lanes 1 and 2) Methylated 8 to 12S mRNA in the absence and presence of m^7pG, respectively; (lanes 3 and 4) unmethylated 8 to 12S mRNA in the absence and presence of m^7pG respectively; (lanes 5 and 6) methylated HMW RNA in the absence and presence of m^7pG, respectively, (lanes 7 and 8) unmethylated HMW RNA in the absence and presence of m^7pG, respectively. The positions of molecular weight markers are shown in the margins. Exposure time for this autoradiograph was 3 weeks.*

incubation. Both HMW RNA and 8 to 12S mRNA's were degraded at similar rates, and the source of the nuclease activity was traced to the creatine phosphokinase preparation. Several lots of creatine phosphokinase were tested, and all were found to contain nuclease activity (12). Although some degradation of HMW RNA and 8 to 12S mRNA was observed in the translation system, over 50% of the HMW RNA sedimented at greater than 18S within 15 min of incubation. The time for initiation and runoff after ATA addition was 10 min (Fig. 4); consequently, under these conditions if there were translation of internal sequence near the 3′ end of the HMW RNA after the initiation of translation occurs at the 5′ end it would be reflected in both the kinetics of runoff (Fig. 4) and in the gel analysis of polypeptides labeled during the runoff (Fig. 5). Furthermore, all polypeptides which are normally synthesized in response to HMW RNA

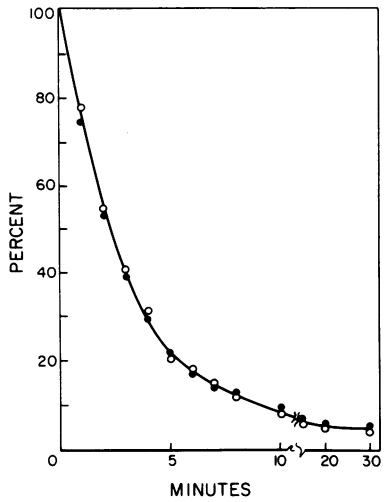

FIG. 4. *Incorporation of amino acids into protein with time after addition of ATA to translation systems programmed by either HMW RNA or 8 to 12S mRNA. The translation of either HMW RNA (40 μg/ml) or 8 to 12S (10 μg/ml) mRNA was initiated and allowed to proceed for 4 min in the absence of added leucine, at which time ATA was added to a final concentration of 75 μM. [^{3}H]leucine was then added to portions of the ATA-inhibited reactions at zero time, i.e., with the ATA, or at the times indicated in the graph after ATA addition. Elongation was allowed to continue for a total of 30 min after the addition of [^{3}H]leucine for each time point. [^{3}H]-leucine incorporation into protein was determined by the method of Mans and Novelli (21). The level of [^{3}H]leucine incorporation resulting when [^{3}H]leucine was added with ATA (zero time) is considered 100% incorporation as presented on the ordinate. Symbols: (●) runoff kinetics for 8 to 12S mRNA; (○) runoff kinetics for HMW RNA.*

when initiation of translation is not limited are also synthesized when the initiation step is inhibited 4 min after the start of translation (Fig. 5B). Given the kinetics of runoff (Fig. 4), this result strongly suggests that none of the expressed coding sequences in the population of HMW RNA are located exclusively at internal sites of a polycistronic RNA molecule. From the combined results, we conclude that only one coding sequence is translated from each HMW RNA and that that sequence is probably located near the 5′-terminal end of the HMW RNA molecule.

Translation of 8 to 12S RNA derived from HMW RNA. Since normally released 8 to 12S mRNA was found to be approximately three- to fourfold more efficient on a weight basis in stimulating protein synthesis than was purified HMW RNA, it was considered important to determine whether 8 to 12S RNA derived from cores containing HMW RNA during incubation in the presence of ATP, GTP, and AdoMet is translated with a similar efficiency as HMW RNA or as normally released 8 to 12S mRNA. Virion-released 8 to 12S mRNA and virion-associated HMW RNA were synthesized as described above. Cores containing HMW RNA synthesized at limiting ATP concentrations were washed twice, and the RNA in one-half of the cores was chased for 60 min in the presence of ATP, GTP, and AdoMet. The three RNA species were then prepared for translation by phenol extraction, chromatography on G-25 Sephadex to remove nucleoside triphosphates and, finally, ethanol precipitation and resuspension in water. It must be stressed that preparation of these RNAs did not include a purification step on sucrose gradients to remove traces of virion-associated 8 to 12S RNA in the HMW RNA preparation as was done for the other experiments reported in this paper. Since the contaminating 8 to 12S RNA is three to four times more efficiently translated than true HMW RNA, even a 10% contamination will significantly decrease the apparent difference in translational efficiencies of these two RNA populations. Sedimentation patterns of the three RNA preparations were examined to determine whether the chase was efficient. There was a slight contamination of the HMW RNA with RNA which sediments in the region of released 8 to 12S mRNA (Fig. 6). As in most experiments, HMW RNA was chased to 8 to 12S RNA and released from the virion with an efficiency of approximately 65%.

Shown in Fig. 7A, B, and C are the saturation curves for the translation of the three RNA species. As expected, the difference in saturation curves for HMW RNA and normally released 8 to 12S mRNA is reduced from that shown in Fig. 1. At low concentrations of RNA, the level of protein synthesis stimulated by derived 8 to 12S RNA is similar to that of normally released 8 to 12S mRNA rather than the virion-associated HMW RNA. This increased specific translatability suggests the processing of HMW RNA into active 8 to 12S mRNA molecules. The saturation curve is unusual, however, in that the level of protein synthesis at the apparent saturation level of RNA is reduced from the levels observed for both the normally released 8 to 12S mRNA and HMW RNA. Furthermore, at higher RNA concentrations, the level of protein synthesis is radically reduced.

This reduction in protein synthesis at higher RNA concentrations suggests the presence of an inhibitor. Recently, the synthesis of a polyadenylated and capped small (4S) RNA species

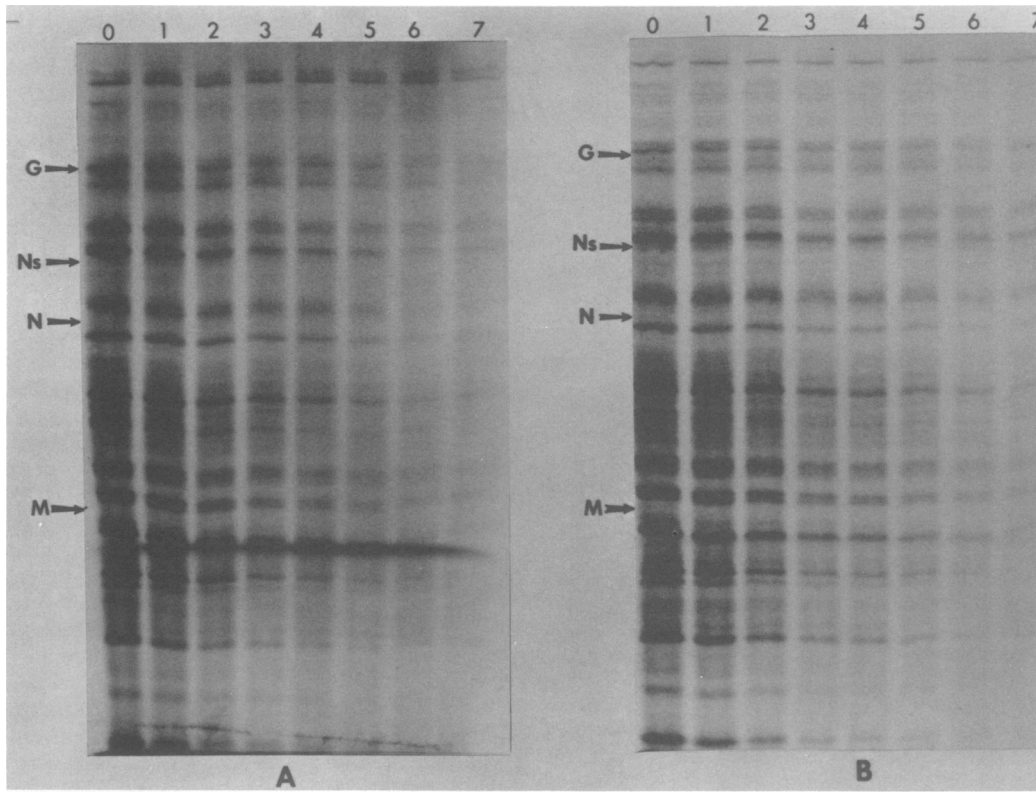

FIG. 5. *Autoradiograph of a SDS-polyacrylamide gel after electrophoresis of polypeptide labeled during a runoff experiment. Conditions for the runoff experiment were identical to those described for Fig. 4, except that [^{3}H]leucine was replaced by [^{35}S]methionine. Each well received an equal volume of the respective reaction mixture, with the zero time sample (lane 1) containing 80,000 cpm. (A) 8 to 12S mRNA; (B) HMW RNA. The numbers at the top of each lane indicate the time after ATA addition that the labeled amino acid was added. The fluorographic procedure of Bonner and Laskey (4) was used for the processing of these gels.*

(polyadenylated leader sequences [PALS]) by vaccinia cores under a variety of conditions has been observed (Paoletti and Lipinskas, manuscript in preparation). If the distortion of the saturation curve for the derived 8 to 12S RNA results from the presence of these small RNA species acting as inhibitors of translation, then addition of a purified fraction of this RNA to released 8 to 12S mRNA should generate a curve similar to that observed in Fig. 7C. As shown in Fig. 7D, E, and F, addition of increasing amounts of PALS did indeed result in an alteration of the saturation curve for 8 to 12S RNA to curves approaching that shown in Fig. 7C.

PALS are synthesized in the ATP-depleted reaction but are released only after restoration of the ATP concentration in the reaction mixture, as was done for the chase in Fig. 6. Thus, PALS are normally present in the virion-associated HMW RNA population as well as in the population of derived 8 to 12S RNA yet, inexplicably, high concentrations of HMW RNA do not show the inhibition of protein synthesis observed with high concentrations of derived 8 to 12S RNA.

Analysis of the translation products synthesized in response to the derived 8 to 12S RNA showed the appearance of no new bands, no increase in the synthesis of a subset of polypeptides also synthesized in response to virion-associated HMW RNA, and no synthesis of low-molecular-weight protein due to the translation of PALS (data not shown). The translation product directed by virion-associated RNA and derived 8 to 12S RNA were identical and again similar to the product synthesized in response to normally released 8 to 12S mRNA and synthesized in the coupled system.

DISCUSSION

Purified vaccinia virion-associated HMW RNA directs polypeptide synthesis in a wheat germ cell-free translation system. Translation

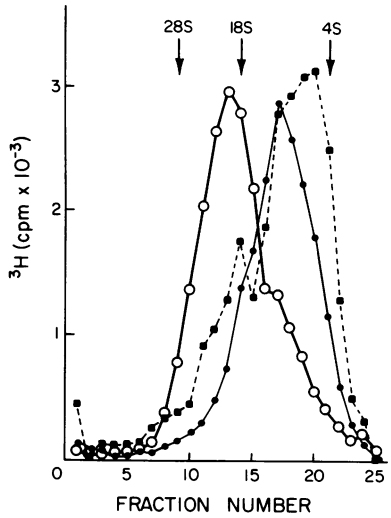

FIG. 6. *Sedimentation profiles of normally released 8 to 12S mRNA, virion-associated RNA synthesized at limiting concentrations of ATP, and released 8 to 12S RNA derived from the virion-associated RNA during a chase in the presence of ATP, GTP, and AdoMet. Virion-released 8 to 12S RNA (●) was purified from a virus-free supernatant after 40 min of synthesis. Virion-associated HMW RNA (○) was synthesized in an ATP-depleted reaction as described (29a) from virus pelleted from a polymerase reaction. Released 8 to 12S mRNA (■) derived from the virion-associated RNA during a chase in the presence of ATP, GTP, and AdoMet was purified from a virus-free supernatant after 60 min of chase. [^{3}H]UTP specific activity was maintained constant in all reactions to monitor the synthesis of RNA.*

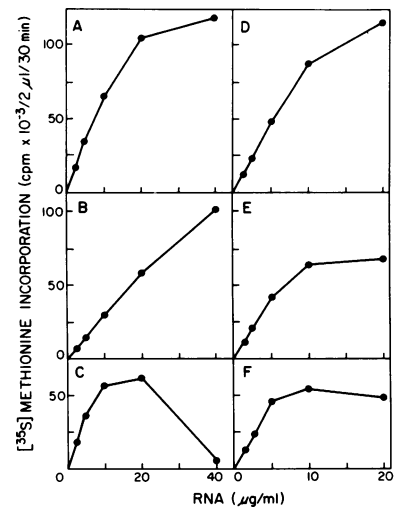

FIG. 7. *Stimulation of polypeptide synthesis in a wheat germ cell-free translation system in response to normally released 8 to 12S mRNA, virion-associated RNA made at limiting concentration of ATP, and released 8 to 12S RNA derived from the virion-associated RNA during a chase in the presence of ATP, GTP, and AdoMet. Translation of each species of RNA synthesized as described in the legend to Fig. 6 was performed as described for Fig. 1. (A) normally released 8 to 12S mRNA; (B) virion-associated RNA made at limiting ATP concentrations; (C) released 8 to 12S RNA derived from virion-associated RNA during a chase in the presence of ATP, GTP, and AdoMet. (D), (E), and (F) show the effect of adding increasing concentrations of small polyadenylated and capped RNAs (PALS) on the translation of normally released 8 to 12S mRNA. The final concentrations of PALS were 0, 5, and 10 µg/ml, respectively, for (D), (E), and (F). PALS were synthesized in the presence of 20 µg of actinomycin D per ml and purified by phenol extraction and selection on poly(U)-Sepharose. Details of the synthesis and characterization of PALS will be presented in a future communication.*

products synthesized in response to HMW RNA are qualitatively identical and quantitatively similar to the translation products synthesized in response to virion-released 8 to 12S mRNA. Efficient initiation of HMW RNA translation requires that these large transcripts be capped at the 5′ terminus. Initiation of translation at internal initiation sites on the HMW RNA molecules seems not to occur. Finally, only one coding sequence on each HMW RNA molecule is translated, and that sequence is probably located very near the 5′-end of the molecule.

An interpretation of these results in terms of the origin and biological significance of the vaccinia HMW RNA is somewhat limited by the sheer complexity of this large DNA virus. The size of the vaccinia genome is estimated at 123 $\times 10^6$ daltons (10). Based on the calculation that vaccinia mRNA has an average length of 1,100 nucleotides (5) and the assumption that only non-complementary regions of the DNA are transcribed, there is a minimal potential within the vaccinia genome for the expression of between 160 and 180 different gene products. Although transcriptional complexity studies indicate that essentially all of the viral genome is transcribed in vitro (6, 28), abundancy measurements do suggest that a small proportion of the genome is represented by the majority of the in vitro-synthesized RNA (6). In this regard, the translation products synthesized in the coupled transcription-translation system programmed by vaccinia virus particles (7, 30, 31) or in cell-free systems programmed by purified 8 to 12S mRNA (7) (Fig. 2) have been resolved into 30 to 40 discrete polypeptides on one-dimensional polyacrylamide gels and approximately 60 polypeptides on two-dimensional gels.

Considerable evidence has accumulated which

is consistent with a precursor-product relationship between the vaccinia virion-associated HMW RNA and the virion-released 8 to 12S mRNA (25–29a). Nevertheless, there are several observations which are, at present, difficult to reconcile with this hypothesis. For example, only transcripts having 5′-di- or triphosphate termini, but not 5′-hydroxyl or -monophosphate termini, which would be produced by cleavage of a HMW RNA precursor, are capped by the virion-associated guanylytransferase (2, 9, 22, 23). Although the elucidation of the mechanism by which caps are formed allows the elimination of several possible pathways which could be envisioned for processing of a large transcript, several alternative pathways, e.g., a mechanism analogous to gene splicing in adenovirus (3, 8, 17) or simian virus 40 (1) have not yet been ruled out.

A second observation which is difficult to understand in terms of a processing mechanism comes from recent studies which examined the effect of UV irradiation on the expression of vaccinia gene products in a coupled transcription-translation system (7, 30). The results of these studies indicate that each translationally functional mRNA produced in vitro by vaccinia cores is synthesized from individual promotor sites and is not derived from large polycistronic transcripts. In this case, it is conceivable that the polypeptides synthesized in the coupled system are coded for by RNA sequences located at or near the 5′ terminus of a few large transcriptional units and that sequences downsteam from the 5′ end of these large transcripts are not expressed because of a lesion in the normal processing mechanism under in vitro conditions. Consistent with this possibility are the data presented in Fig. 2, 4, and 5 which suggest that only one sequence per HMW RNA molecule is translated, that this sequence is probably located at or near the 5′ end of the HMW RNA molecule, and that the sequences expressed in response to HMW RNA are the same as those expressed both in the cell-free translation system in response to 8 to 12S mRNA and in the coupled system. However, if one considers the possibility that virion-released 8 to 12S mRNA is derived from a set of polycistronic HMW RNAs by an in vitro processing mechanism in which only the sequences derived from the 5′ end of the large transcript are translationally functional, whereas the processed sequences derived from internal regions of the HMW RNA precursor are translationally nonfunctional, then one would predict that the translational activity of the HMW RNA and the derived 8 to 12S RNA would be identical on an equal weight basis. As shown in Fig. 1 and in Table 1, this is not the case; 8 to 12S mRNA is approximately three- to fourfold more active in stimulating protein synthesis in the cell-free system than a comparable amount of HMW RNA. Thus, there is no evidence for the buildup of translationally inactive RNA sequence as a result of faulty processing. Furthermore, if only the 5′-terminal sequence of a polycistronic HMW RNA precursor were processed in vitro to yield a translationally active molecule, but several translationally active mRNA molecules were derived from each HMW RNA precursor in the infected cell, where processing would proceed normally, then there should be major differences in the polyacrylamide gel patterns of the translation products synthesized in a cell-free system in response to either polysomal mRNA from vaccinia-infected cells or in response to in vitro-synthesized 8 to 12S mRNA. In fact, polyacrylamide gel patterns of the translation products directed by either in vitro-synthesized vaccinia mRNA or RNA isolated from polysomes of vaccinia-infected cells are quite similar (J. Cooper and B. Moss, personal communication).

Perhaps each HMW RNA is the precursor of only one 8 to 12S mRNA molecule and the remainder of the HMW RNA sequences are either degraded or remain virion associated. In this regard, the results of experiments in which HMW RNA was preferentially (27) or exclusively (29a) labeled and then cleaved to RNA in the 8 to 12S size range suggests that the majority of this labeled HMW RNA is conserved during cleavage; i.e., there is no evidence suggesting the extensive degradation of a portion of the HMW RNA during the cleavage step. Indeed, transcriptional complexity studies have indicated that the same sequences are represented in both the virion-released 8 to 12S mRNA and the virion-associated HMW RNA (28).

Given the difference in efficiency with which equal weights of released 8 to 12S RNA and purified virion-associated HMW RNA direct in vitro protein synthesis (Fig. 1), a determination of whether HMW RNA is processed into functional messenger RNA should be possible by comparing the efficiency of translation of derived 8 to 12S RNA with virion-associated HMW RNA from which it was derived and normally released 8 to 12S mRNA. However, this experiment is plagued by two problems. First, the virion-associated RNA synthesized under limiting ATP concentrations is contaminated with some 8 to 12S RNA which is released upon chase in the presence of ATP and thus becomes part of the population of derived 8 to 12S RNA. While this virion-associated 8 to 12S RNA makes up only a fraction of the total bulk of the

virion-associated RNA, it contributes more to the translational efficiency of the total RNA than to its bulk by a factor of 3 to 4. Thus, the difference in efficiency of the normally released 8 to 12S mRNA and the unpurified total virion-associated RNA will be on the order of 1.5- to 2.5-fold rather than 3- to 4-fold. Second, the presence of inhibitory PALS released under the chase conditions (Paoletti and Lipinskas, manuscript in preparation) adds an additional complication to the interpretation of these data. However, based on the level of incorporation observed at low concentrations of derived 8 to 12S RNA, it would seem that the translational efficiency of the virion-associated RNA does increase during the chase. This result is consistent with the proposal that the presynthesized HMW RNA is processed quantitatively within the virion to functional mRNA.

An alternative explanation for the origin of the HMW RNA is that this RNA results from a lesion in the termination of transcription, i.e., a read-through mechanism. It is possible that faulty termination, which might occur at limiting ATP concentrations in vitro (19, 20, 29a), could result in the synthesis of large transcripts with properties similar to those described for the vaccinia virion-associated HMW RNA. Such large transcripts would be capped at the 5′ end (25). The ratio of molecules having either $m^7G(5')ppp(5')G^m$ or $m^7G(5')ppp(5')A^m$ termini would be the same for the large transcripts resulting from read through and for the correctly terminated 8 to 12S mRNA (25). The 8 to 12S mRNA and the large read-through transcripts would have identical sequence complexity (28). Translation products synthesized in cell-free translation systems in response to the large transcripts would be identical to the translation products synthesized in response to 8 to 12S mRNA (Fig. 2), and the sequences that are translated on the large transcripts would be located at the 5′ end of those molecules (Fig. 4 and 5). Finally, the mechanism of cap formation and the observed effect of UV irradiation on vaccinia gene expression in the coupled transcription-translation systems are not inconsistent with a read-through mechanism. At the same time, a read-through mechanism for the generation of HMW RNA does not explain the consistent observation that prelabeled HMW RNA can be cleaved within the virion core, under appropriate conditions, to RNA in the size range of 8 to 12S (27, 29a) which appears to be translationally functional (Fig. 7). Also, the detection of a vaccinia virion-associated endoribonuclease activity (29) which acts specifically on the HMW RNA but not the 8 to 12S mRNA supports a processing mechanism rather than a read-through mechanism. Additional studies are required to determine whether the vaccinia virion-associated HMW transcripts result from faulty termination of transcription or, a more exciting prospect, they represent precursor transcripts to mature vaccinia mRNA.

Independent of the mechanism by which vaccinia HMW RNA is generated, it is likely that these large transcripts contain more than one gene sequence and, consequently, more than one potential ribosome binding site per molecule. It is interesting, therefore, that translation of these large transcripts is initiated only through a 5′-terminal cap-mediated step and that initiation at internal sites does not occur. These large transcripts should prove useful for studies aimed at understanding the molecular basis of site-specific recognition of mRNA by eucaryotic ribosomes.

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