JOURNAL OF VIROLOGY, June 1967, p. 550-558

Vol. 1, No. 3
Printed in U.S.A.

Metabolic Properties of Early and Late Vaccinia Virus Messenger Ribonucleic Acid

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Received for publication 25 January 1967

In vaccinia-infected cells, 60% of the viral messenger ribonucleic acid (mRNA) was associated with polyribosomes, and the remainder sedimented in a broad peak in the 30 to 74*S* region. The quantity of mRNA in polyribosomes was sharply reduced late in the infectious cycle [9 hr postinfection (PI)] to less than 30% of the 2-hr value. However, protein synthesis proceeded at a nearly constant rate from 2 to 13 hr PI. This ability of small quantities of late mRNA to support as much protein synthesis as do the much larger quantities of early mRNA was not due to an increase in stability, since late mRNA decays with a half-life of 13 min, whereas early mRNA has a half-life of 120 min. A similar decrease in viral mRNA synthesis without an accompanying decrease in viral protein synthesis was observed when deoxyribonucleic acid synthesis is inhibited. In contrast to the rapid decay of the late mRNA which was present in polyribosomes, the mRNA which sedimented in the 30 to 74*S* region remained unchanged even after a 2-hr period of exposure to actinomycin. The rate at which infected cells lose the capacity to synthesize specific viral proteins after exposure to actinomycin D was consistent with the half-life values of early and late mRNA that were observed.

It has been demonstrated that during the cycle of vaccinia virus replication total viral protein synthesis proceeds at a linear rate (9, 13). However, individual viral proteins are synthesized in a sequential manner. Of the proteins which are incorporated into mature virus particles, 85% are made late in the infectious cycle [after 6 hr postinfection (PI)]. The proteins which are synthesized early in the cycle are immunologically distinct from the late proteins, and only a small fraction of these early proteins appear in the viral progeny. Normally, synthesis of the early proteins does not continue beyond 6 hr PI. However, when viral deoxyribonucleic acid (DNA) synthesis is blocked, early protein synthesis is not switched off at 6 hr PI, but continues for at least an additional 6 hr (9). McAuslan (5, 6) made a similar observation with respect to thymidine kinase, an enzyme that is induced in vaccinia-infected cells. During a normal cycle of virus replication, enhanced levels of this enzyme are observed at 2 hr PI, and no further increase in the enzyme level is observed after 6 hr PI. In the absence of DNA synthesis, the quantity of enzyme continues to increase for 24 hr. Thymidine kinase synthesis is coded for by a metabolically stable messenger ribonucleic acid (mRNA). The repression of enzyme synthesis that is observed at 6 hr PI is thus not the result of a breakdown of mRNA, but rather results from the de novo synthesis of both RNA and protein.

In the present investigation, we have measured the metabolic stability and the quantity of early and late viral mRNA at different times in a normal cycle of replication. The effect of viral DNA replication on the synthesis of viral mRNA has also been determined.

MATERIALS AND METHODS

Conditions for cell growth and for virus infection are described in a previous paper (9).

Preparation of cytoplasmic extracts. Cells were collected by low-speed centrifugation, washed with Eagle's spinner medium, and suspended in RSB [0.01 M tris(hydroxymethyl)aminomethane chloride at *p*H 7.4 containing 0.01 M KCl and 1.5 mM $MgCl_2$]. After standing at 0 C for 5 min, cells were broken with 5 to 10 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1,800 rev/min for 10 min at 0 C to remove nuclei and unbroken cells.

Preparation of cytoplasmic RNA. Sodium dodecyl sulfate (SDS) was added to cytoplasmic extracts at a final concentration of 0.35%. Samples were then extracted two times with an equal volume of phenol at 0 C (10), and RNA was precipitated from the aqueous phase by addition of 2 volumes of alcohol. RNA was dissolved in 1 ml of acetate buffer (0.01 M sodium acetate at *p*H 5.1 containing 0.1 M NaCl and

1 mM $MgCl_2$) and passed through a column (2 by 15 cm) of Sephadex G-25. RNA was isolated from the appropriate fractions by addition of 2 volumes of alcohol. The RNA was collected by centrifugation and then redissolved in 1 ml of acetate buffer.

Sedimentation analysis. The RNA samples were layered over 25 ml of a linear 5 to 20% sucrose gradient in acetate buffer, and were centrifuged at 25,000 rev/min for 12 hr in a Spinco SW-25.1 rotor.

To examine the association of mRNA with cytoplasmic components, cytoplasmic extracts were added to sodium deoxycholate at a final concentration of 0.25%. These solutions were layered over 23 ml of a linear 5 to 20% sucrose gradient in RSB with a 5-ml "cushion" of 70% sucrose in RSB. The samples were centrifuged at 25,000 rev/min for 4.33 hr in an SW-25.1 rotor. The sample tubes were punctured, the optical density of the gradient was monitored with a Gilford recording spectrophotometer, and 1-ml fractions were collected automatically with a fraction collector.

Assay of radioactivity. A 1-ml amount of a 0.1% solution of yeast RNA was added to each of the samples from the sucrose gradient, and the fractions were precipitated by the addition of an equal volume of 5% trichloroacetic acid. To determine the quantity of either ^{14}C or ^{32}P that was present, the precipitated RNA was collected by centrifugation, dissolved in 0.3 N NH_4OH, and plated on stainless-steel planchets. Radioactivity was determined in a Nuclear-Chicago low-background counter (Nuclear-Chicago Corp., Des Plaines, Ill.) In experiments involving the simultaneous counting of two isotopes, the precipitated RNA was collected on Gelman GA-6 filters (Gelman Instrument Co., Ann Arbor, Mich.), and the filters were washed with 5% trichloroacetic acid. Filters were dried overnight at 25 C, and were then incubated for 30 min with 1 ml of 0.1 N hyamine in methanol to effect solution of the RNA contained on the filters. After addition of 15 ml of Liquifluor, ^{14}C and ^{3}H counts were determined in a Nuclear-Chicago liquid scintillation spectrometer.

Examination of viral proteins by agar diffusion. The procedure for examination of proteins by agar diffusion and radioautography and the activity of the antiserum have previously been described (9). The antiserum was a gift of G. Appleyard. This antiserum is able to react with both early and late viral proteins.

RESULTS

Effect of an inhibition of cellular DNA synthesis on the synthesis of cytoplasmic RNA in uninfected cells. The uninfected cultures used in this experiment were manipulated in the same way as infected cultures. HeLa cells from a suspension culture were collected by centrifugation and resuspended at 5×10^6 cells per ml in Eagle's medium containing uridine (3×10^{-5} M) and 5-fluorodeoxyuridine (FUDR, 10^{-6} M). After 1 hr at 37 C, the cells were collected by low-speed centrifugation, washed one time with the same medium, and then resuspended at 4×10^5 cells per ml in this medium. The culture was divided, and thymidine at a final concentration of 10^{-6} M was added to one part. The concentration of thymidine that was added reverses the inhibitory effects of FUDR and allows DNA synthesis to proceed at optimal rates for at least 6 hr (14), whereas, in its absence, DNA synthesis is completely inhibited. The two cultures were exposed to $^{32}PO_4^{-3}$ for 3 hr, the cytoplasmic RNA was isolated by phenol extraction, and this RNA was further purified by chromatography on Sephadex G-25 (10). Each sample of RNA was then fractionated by centrifugation in a 5 to 20% sucrose gradient. The inhibition of DNA synthesis had no effect on either the quantity of newly synthesized cytoplasmic RNA or its distribution in a sucrose density gradient (Fig. 1C and D). The nucleotide base ratios (12) of the newly synthesized 4S, 16S, and 28S RNA's were unaffected by an inhibition

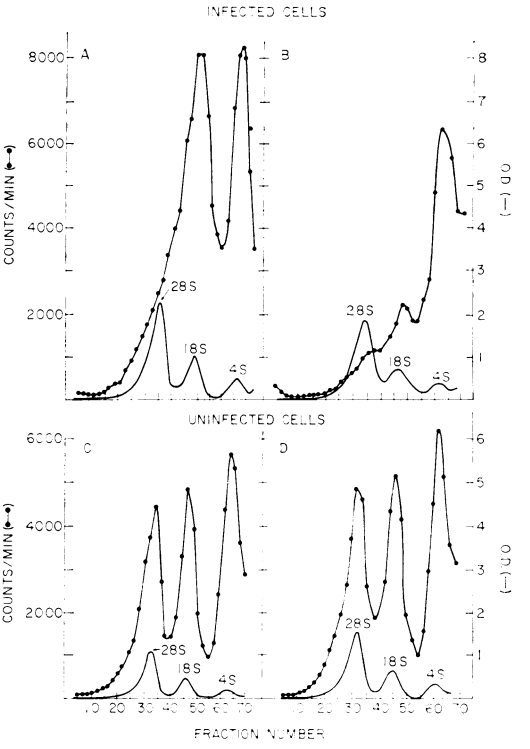

FIG. 1. *Sucrose density gradient analysis of cytoplasmic RNA extracted with phenol-SDS. RNA was isolated and purified as described in the experimental section. The RNA was fractionated in a 5 to 20% linear sucrose gradient, and the gradient was monitored for radioactivity and optical density. (A) Infected culture exposed to $^{32}PO_4^{-3}$ from 1 to 4 hr PI; (B) infected culture plus FUDR exposed to $^{32}PO_4^{-3}$ from 1 to 4 hr PI; (C) uninfected culture exposed to $^{32}PO_4^{-3}$ for 3 hr; (D) uninfected culture plus FUDR exposed to $^{32}PO_4^{-3}$ for 3 hr.*

of DNA synthesis, and were identical with the values previously reported (10). In agreement with these findings are previous studies which demonstrated, by use of colorimetric procedures, that the amount of cytoplasmic RNA that was synthesized in a 24-hr period was unaffected by an inhibition of DNA synthesis (8).

Effect of inhibition of DNA synthesis on the synthesis of viral mRNA. Cells were manipulated in the same manner as described above, except that 20 to 40 plaque-forming units (PFU) per cell of vaccinia virus (strain WR) was added after the cells were resuspended at 5×10^6 cells per ml. Under these conditions, all cells in the culture were infected. The inhibitor, FUDR, which was present during the period of virus adsorption and when unadsorbed virus was removed, did not affect either the rate of virus adsorption or virus eclipse. After the culture was divided, thymidine (10^{-6} M) was added to one part. This permits vaccinia virus to replicate with normal kinetics, whereas in the absence of added thymidine there is no infectious virus formed (7). The pattern of newly synthesized RNA in infected cultures exposed to $^{32}PO_4^{-3}$ from 1 to 4 hr PI is shown in Fig. 1A. As described previously (10), in the infected culture the newly synthesized mRNA peaks in the 16*S* region of the gradient, and there is a sharp reduction in the amount of newly formed RNA in the 28*S* region as compared with an uninfected control. The nucleotide composition of newly synthesized RNA in different parts of the gradient demonstrates that all of the RNA present that is larger than 9*S* has a nucleotide composition similar to viral DNA [guanine plus cytosine (G + C) = 34%]. An inhibition of viral DNA synthesis caused a marked reduction of viral mRNA synthesis in the infected culture (Fig. 1B). At 1 to 4 hr PI, there was an 80% suppression in the incorporation of $^{32}PO_4^{-3}$ into viral mRNA as compared with infected cultures in which DNA replication occurs. In a normal cycle of infection, synthesis of cellular RNA continues in the nucleus for the first 7 hr of the infectious cycle. However, as a consequence of virus infection, this newly synthesized cellular RNA is excluded from the cytoplasm (10). The sharp reduction in the quantity of newly synthesized cytoplasmic RNA which was observed (Fig. 1B) when viral DNA replication was inhibited demonstrates that exclusion of nuclear RNA from the cytoplasm also occurs under these conditions.

Association of viral mRNA with cytoplasmic components. Infected cells were labeled with uridine-2-^{14}C for 10-min periods at different times PI. Parallel experiments were carried out in the presence of 10^{-6} M FUDR and also with uninfected cells. The cells were broken in RSB, and the extract, after treatment with sodium deoxycholate, was layered onto a 5 to 20% linear sucrose gradient containing a cushion of 70% sucrose (15). Centrifugation was carried out at 25,000 rev/min for 4.33 hr. With this procedure, polyribosomes accumulate in the 70% sucrose. The ribosomal subunits, with sedimentation values of 45*S* and 60*S* (1), and ribosomes, with a sedimentation value of 74*S*, sediment as distinct

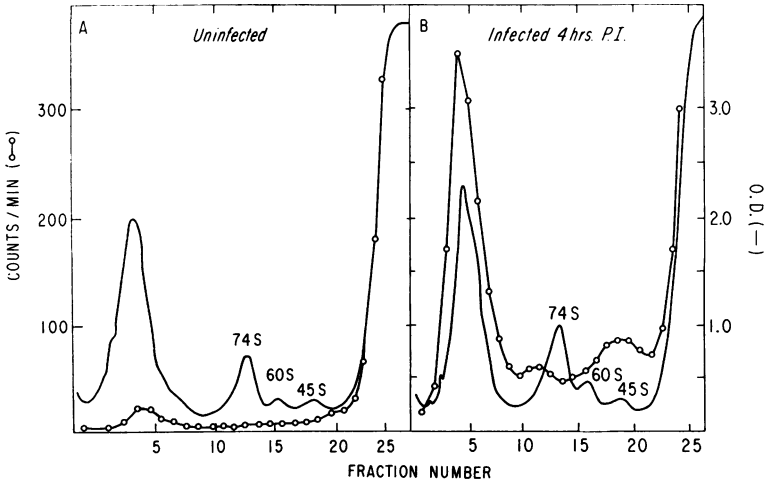

FIG. 2. *Association of viral mRNA with cytoplasmic components during a normal cycle of virus replication. The cytoplasmic fractions were prepared as described in the experimental section. Samples were layered onto a 5 to 20% sucrose gradient with a 70% sucrose "cushion," and were centrifuged for 4.33 hr at 25,000 rev/min at 0 C. (A) Uninfected cells were labeled for 10 min with 5 μc of uridine-2-^{14}C. (B) Infected cells were labeled for 10 min with 5 μc of uridine-2-^{14}C at 4 hr PI.*

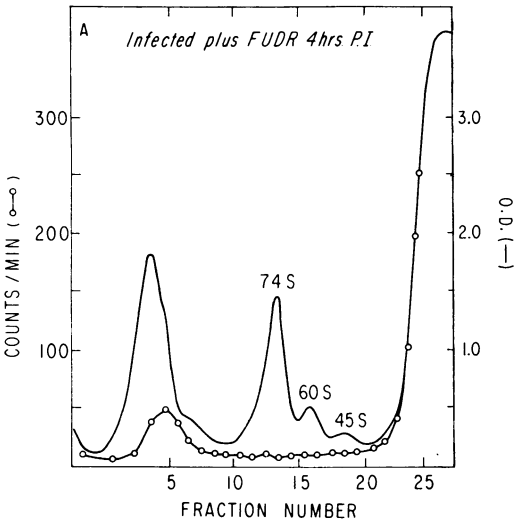

FIG. 3. *Association of viral mRNA with cytoplasmic components in the absence of viral DNA replication. See legend to Fig. 2. FUDR-inhibited infected cells were labeled for 10 min with 5 μc of uridine-2-^{14}C at 4 hr PI.*

peaks. By use of a 10-min period for isotope incorporation, very low levels of newly synthesized RNA were detected in the cytoplasm of the uninfected cells (Fig. 2A). The distribution of viral mRNA in such a gradient is seen in Fig. 2B. A pattern similar to that shown in Fig. 2B was observed in the infected cultures from 3 hr PI on, although the quantity of mRNA in the polyribosome region varied at the different time periods as described below. Radioactivity corresponding to newly synthesized viral mRNA was distributed in two regions of the gradient (Fig. 2B). Between 60 and 70% of the labeled RNA was found in the polyribosome region of the gradient (fractions 1 to 9). Approximately 80% of the remaining viral mRNA was distributed in a broad heterogeneous peak in the 30 to 74*S* region of the gradient (fractions 14 to 22). The newly synthesized RNA's which were isolated from either polyribosomes or the 30 to 74*S* region behaved in an identical manner in a sucrose density gradient. The newly synthesized RNA's have sedimentation constants of 16*S* or greater, and both have nucleotide compositions similar to vaccinia DNA (15).

The distribution of the viral mRNA that was synthesized at 4 hr PI in the absence of DNA replication is shown in Fig. 3. A similar pattern was seen at 2, 6, and 9 hr PI. In the absence of DNA synthesis, the quantity of viral RNA found in both the 30 to 74*S* region and in polyribosomes was sharply reduced.

Quantity of mRNA in polyribosomes. It was

TABLE 1. *Quantity of viral mRNA in polyribosomes of cells infected with vaccinia virus*

Time PI	Quantity of viral mRNA in polysomes			
	Total counts/min in polyribosome peak		Relative amt of mRNA in polyribosomes[a]	
	Infected	Infected, FUDR-inhibited	Infected	Infected, FUDR-inhibited
hr				
2	1,850	560	100.0	30.3
4	1,790	238	96.7	12.9
6	1,673	258	90.4	13.9
9	514	218	27.8	11.8

[a] The quantity of mRNA found in the infected culture at 2 hr (1,850 counts/min) is set at 100, and the other values are expressed as the percentage relative to this value.

shown previously that it is the polyribosomes which become labeled after a 1-min period of exposure in vivo to radioactive amino acids (15), and in which newly synthesized antigenically identifiable protein is found (11). It is logical to conclude that functioning mRNA which is present in the infected cells is present in polyribosomes. The quantity of mRNA in polyribosomes was determined after a 10-min period of exposure to uridine-2-^{14}C at different times in a normal cycle of infection, and when infection was carried out in the absence of DNA replication (Table 1). In a normal cycle of replication, a reduction in the quantity of mRNA in polyribosomes was observed at 9 hr PI. This decrease of 60 to 70% in the quantity of mRNA in polyribosomes is not reflected in a decreased rate of viral protein synthesis, which proceeds at nearly the same rate throughout the infectious cycle (9, 13). The inhibition of viral DNA synthesis results in an inhibition of viral mRNA synthesis even at the earliest time period examined, in agreement with the findings shown in Fig. 1B. Although a 70% inhibition of viral mRNA synthesis is noted at 2 hr PI, at this time no depression in the rate of viral protein synthesis is observed (9, 13). This is also true at 4 hr PI when there is an 87% decrease in the quantity of mRNA in polyribosomes. If the level of mRNA determines the quantity of viral protein that is synthesized, a sharp reduction in viral protein synthesis would have been expected.

Metabolic stability of mRNA. Protein synthesis continued at a constant rate throughout the infectious cycle in spite of the sharp reduction in the quantity of mRNA that was present in polyribosomes late in the infectious cycle. These findings might be explained if there were a prior

accumulation of mRNA in polyribosomes, or if mRNA had a significantly longer half-life late in the infectious cycle.

In an initial experiment, it was established that, when actinomycin D was added at 6 hr PI, the loss of ribosomes from polyribosomes exactly paralleled the loss of pulse-labeled mRNA from polyribosomes. The loss of ribosomes from polyribosomes, which was measured by a decrease in optical density of the polyribosome peak, was equal to the increase in optical density of the single ribosome peak. To determine the loss of ribosomes from polyribosomes, a culture was prelabeled by growing cells for two generations in the presence of adenine-*8*-^{14}C to label ribosomal RNA. This culture was then infected with vaccinia virus, and three replicate cultures were set up. Uridine-*5*-^{3}H (1 μc/ml) was added to one culture at 1.75 hr PI, and to the other cultures at 5.75 and 8.75 hr PI. At 15 min after the addition of uridine-*5*-^{3}H, 100-ml samples of the cultures were poured over frozen, crushed media to stop incorporation. At the same time, actinomycin D (5 μg/ml) was added to the remainder of each culture. At 5, 15, 30, 60, 120, and 480 min after addition of actinomycin D, 100-ml samples were poured over frozen media. Cytoplasmic extracts were prepared in RSB and were sedimented through a 5 to 20% sucrose gradient with a 70% sucrose "cushion" for 4.33 hr at 25,000 rev/min. Optical density and acid-precipitable ^{14}C and ^{3}H counts were determined for each of the total polyribosomal and ribosomal fractions. The results are expressed in terms of a constant amount of total ribosomes to correct for variable recoveries. In Fig. 4, the amount of unstable polyribosomes present is plotted on a logarithmic scale against time after addition of actinomycin D. As noted below, 30 to 40% of those polyribosomes present at 6 hr PI were not broken down. The polyribosomes, at all three time periods, decayed exponentially. The polyribosomes at 6 and 9 hr PI decayed with a 5-min half-life. In contrast to the rapid rate of degradation of late polyribosomes, polyribosomes at 2 hr PI have a half-life of 120 min; that is, they are degraded at only 4% the rate observed for late polyribosomes. At 6 and 9 hr PI, the loss of pulse-labeled mRNA (^{3}H counts) from polyribosomes coincided with the decay of polyribosomes. The 5-min half-life of late mRNA observed here was shorter than is usually observed. In four separate experiments at 6 hr PI, half-life values of 5, 12, 13, and 22 min were observed. The stability of early and late mRNA was the opposite of what we would have predicted, based on the high efficiency of late mRNA as a template for viral protein synthesis as compared with early mRNA. The rapid decay of mRNA in polyribosomes late in the infectious cycle also excludes the possibility of any significant prior accumulation of mRNA in the polyribosomes. These results demonstrate that neither the quantity of mRNA nor its metabolic stability is the sole mechanism which controls the rate of protein synthesis.

The stability of 30 to 74*S* mRNA in the presence of actinomycin D can be demonstrated as being clearly distinct from mRNA in poly-

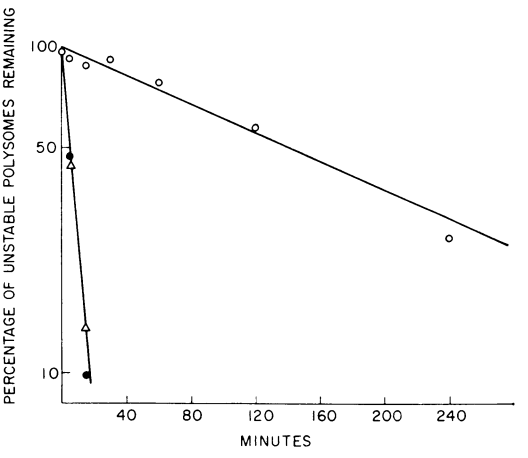

FIG. 4. *Decay of unstable polyribosomes in the presence of actinomycin D. Actinomycin D was added to infected cultures at 2, 6, and 9 hr PI. At various times thereafter, the amount of polyribosomes present was determined as described in the text. The unstable polyribosomes were defined as the polyribosomes present at time t minus those present after five to six half-periods (8 hr for the 2 hr PI samples and 30 min for the 6 and 9 hr PI samples). Symbols:* ○, *2 hr PI;* ●, *6 hr PI;* △, *9 hr PI.*

TABLE 2. *Differential stability of viral mRNA in polyribosomes and 30 to 74S RNA*[a]

Time after the addition of actinomycin D	Quantity of mRNA in polyribosomes	Percentage of zero value	Quantity of 30 to 74*S* RNA	Percentage of zero value
min	*counts/min*		*counts/min*	
0	6,440	100	2,740	100
5	6,060	94	2,610	95
15	4,400	68	2,900	106
30	3,130	48	3,000	110
60	2,230	35	2,610	95
120	2,050	32	2,800	102

[a] Uridine-2-^{14}C (24 mc/mmole) was added to an infected culture at 5 hr PI. At 6 hr PI, actinomycin D was added at a final concentration of 5 μg/ml. At various times thereafter, samples of the culture were poured over frozen media to stop the reaction. Cytoplasmic extracts were prepared, and analyzed by sucrose gradient centrifugation.

ribosomes. Infected cells were labeled for 1 hr with uridine-2-^{14}C at 6 hr PI. At the time that the first sample was harvested, actinomycin D was added to the remainder of the culture. At 5, 15, 30, 60, and 120 min after addition of the antibiotic, 100-ml samples were poured onto crushed, frozen media. The cells were collected by centrifugation, and the cytoplasmic extracts were prepared in RSB and analyzed by sucrose gradient centrifugation. The rate of degradation of viral mRNA in the 30 to 74S region and in polyribosomes is shown in Table 2. The mRNA in polyribosomes was degraded rapidly after the addition of actinomycin D. However, only 60 to 70% of the mRNA in polyribosomes decayed at this rate. The remainder was stable in the presence of actinomycin D. Phenol-SDS was used to extract the rapidly labeled RNA from the polyribosomes that remained after a 2-hr exposure to actinomycin D. The base composition and sedimentation characteristics of this RNA were identical with that of RNA extracted from polyribosomes of infected cells that were not exposed to actinomycin D (15).

To determine the nature of the viral mRNA in the polyribosomal region that is not degraded after exposure to actinomycin D, material in the polyribosomal region remaining after a 2-hr exposure to actinomycin D was collected from the 5–20–70% sucrose-RSB gradient and dialyzed against RSB. Then material was the resedimented through a 10 to 30% sucrose-RSB gradient for 2 hr at 25,000 rev/min. The pattern of radioactivity was the same as that obtained from polyribosomes of infected cells that were treated in a similar manner but had not been exposed to actinomycin D.

During the 120-min exposure to the antibiotic, no decrease in the quantity of mRNA in the 30 to 74S region of the gradient was observed. We previously reported that infected cells exposed to actinomycin D from 6 to 7.5 hr PI are unable to incorporate labeled amino acids into protein (15). Thus, both the 30 to 74S mRNA and the mRNA in those polyribosomes which do not decay in the presence of actinomycin D do not function to support protein synthesis.

Half-lives of viral RNA associated with the synthesis of specific viral proteins. The half-life values reported above for early and late mRNA reflect the rates of decay of the total polyribosome mRNA populations which exist at these times. A procedure has been described for the examination of viral proteins by agar diffusion and radioautography (9). This procedure can be used to determine the half-lives of mRNA associated with the synthesis of specific viral proteins.

A series of infected replicate cultures was established. At 2 hr PI, one culture was labeled for 10 min with uniformly ^{14}C-labeled L-phenylalanine. At the same time that the first culture was harvested, actinomycin D (5 μg/ml, final concentration) was added to six other cultures. At different times after the addition of actinomycin D, the cells were pulsed for 10 min with uniformly ^{14}C-labeled L-phenylalanine. For each sample, the 10-min uptake of isotope was terminated by pouring the cells onto crushed, frozen medium. The cells were collected washed once with cold medium; after centrifugation, the pellets were resuspended in 1 ml of 0.85% NaCl. After disruption of the cells, the samples were used for agar diffusion and radioautography. The same experiment was also carried out at 6 hr PI. The results (Fig. 5) relate to protein synthesis that occurred at 6 hr PI. The antiserum was diluted so that precipitin lines were only obtained from the principal viral proteins. With a short period of exposure to the X-ray film, only one actively synthesized protein was observed at this time. The ability of the cell to synthesize this protein was lost rapidly, and, by 50 to 60 min after the addition of actinomycin D, the infected cell almost completely lost this capacity. This observation is consistent with the finding that at this time the average value for the half-life of mRNA is 13 min. When the same experiment was performed with the early proteins that are labeled at 2 hr PI, the results were in marked contrast to the result noted above. The early proteins were labeled with almost equal intensity, even at 90 min after the addition of actinomycin. This confirms the finding that early viral mRNA is considerably more stable than late mRNA.

DISCUSSION

The vaccinia virus system is particularly suitable for the type of study described in the present paper, since it permits the study of viral mRNA in the absence of newly synthesized cellular RNA. This is the consequence of the cytoplasmic site of replication of the virus as contrasted to the nuclear site of cellular RNA synthesis, and of the exclusion of newly synthesized species of cellular RNA from the cytoplasm of infected cells (10).

There is a pronounced reduction in the rate of synthesis of viral mRNA at the later stages of virus replication as measured by the rate of total cytoplasmic RNA synthesis (10), or by the quantity of newly synthesized viral mRNA that is found in polyribosomes. The reduction in the quantity of polyribosomes at the later times in the infectious cycle is not accompanied by a change in the rate of viral protein synthesis (9,

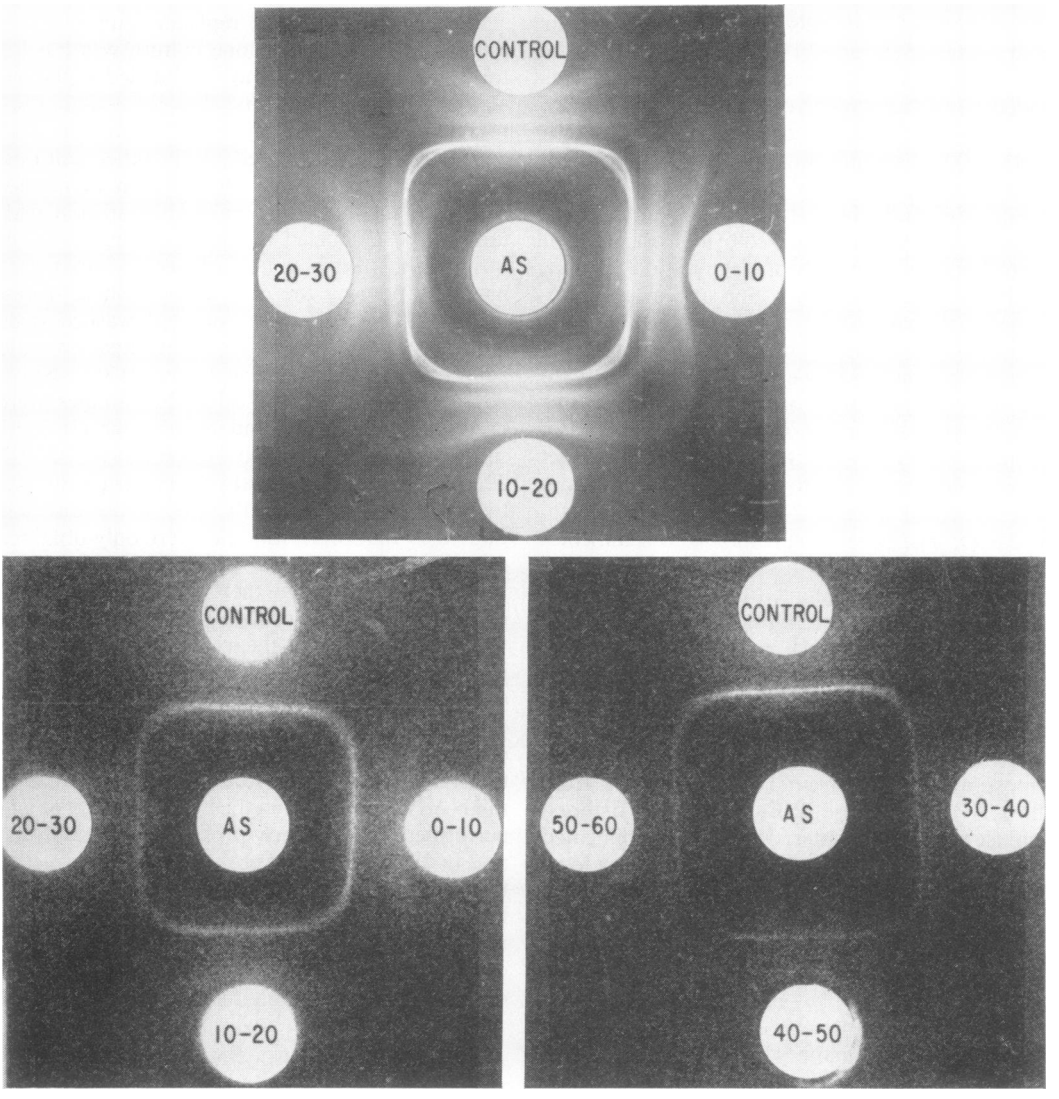

FIG. 5. *Ability of infected cells to synthesize a specific viral protein after exposure to actinomycin D. The infected cultures were in a medium containing 0.01 mM phenylalanine; ^{14}C-phenylalanine (0.1 μc/ml) was added to one infected culture (control) at 6 hr PI. After 10 min, the uptake of isotope was terminated by pouring the infected cells onto crushed, frozen medium. Also, at 6 hr PI, actinomycin D (5 μg/ml, final concentration) was added to six replicate infected cultures. These cultures were similarly exposed to isotope for 10-min periods at various times thereafter. Extracts for agar diffusion were prepared as described in the text. Agar diffusion was carried out at 25 C for 3 days on microscope slides. The slides were washed for 48 hr with 0.85% NaCl, and then with water for 1 hr to remove nonspecific proteins. The slides were then stained with ponceau S, and were used to make enlargements. The top illustration is a photographic enlargement of the stained diffusion pattern for the control and three of the samples that were treated with actinomycin D. The particular 10-min period after the addition of actinomycin D during which each culture was labeled is shown in the appropriate circle. This slide was then placed in contact with Ansco high-speed X-ray film for 3 days. The film was developed, and the radioautograph was used to make a photographic enlargement. This is shown in the bottom, left illustration. The radioautograph obtained from the same control, as well as the three remaining samples, is shown in the bottom, right illustration. The stained protein pattern for this slide is not shown, since it was identical with the stained pattern that is illustrated.*

13). The RNA associated with polyribosomes early in the infectious cycle is relatively stable with a half-life of 120 min as compared with late mRNA which decays with a half-life of 13 min. In the presence of actinomycin D, the cells lose the capacity to synthesize specific viral proteins

at a rate that is in agreement with these half-life values of mRNA. The lability of late mRNA contrasts with the ability of small quantities of this mRNA to act as effectively as a template for viral protein synthesis as do larger quantities of of stable, early mRNA. Evidence has been presented that new species of transfer RNA (t-RNA) are synthesized in cultures infected with herpes simplex virus, and that these tRNA species are coded for by the viral genome (17). The synthesis of tRNA at early and late time periods in the vaccinia infectious cycle with altered codon recognition sites is one possible basis for a variable efficiency of mRNA.

When vaccinia virus DNA replication is blocked, synthesis of early proteins continues beyond the time at which their synthesis is normally switched off (5, 6, 9). A similar observation has been made for the early enzymes induced in *Escherichia coli* infected with T-even bacteriophage (2). McAuslan (5, 6) demonstrated that repression of one early enzyme (thymidine kinase) required the de novo synthesis of both RNA and protein. Inhibition of thymidine kinase formation did not result from degradation of its mRNA, since this RNA was shown to be highly stable in the presence of actinomycin D. Similarly, our own results show that early mRNA is not degraded rapidly in the presence of actinomycin D.

Data have been published by Joklik and Becker (4) which were interpreted as evidence that the vaccinia mRNA in the 30 to 74*S* region of the gradient is bound to a 40*S* ribosomal subunit and serves as a precursor of polyribosomes. In those experiments, the RNA was considered a precursor when transfer of label from mRNA in the "40 to 45*S* region" to polyribosomes was observed in the presence of actinomycin D. In their experiment, an increase of 420 counts in the "product" could clearly not have resulted from the "precursor," which decreased by only 170 counts. The transfer of isotope into polyribosomes in the presence of actinomycin D is inconsistent with our observation that late mRNA in polyribosomes decays at a logarithmic rate with a half-life of 13 min, and also with a similar finding by Joklik (3) that 50% of the polyribosomes are degraded within 30 min in the presence of actinomycin D. It is also inconsistent with the finding that no decrease is observed in the quantity of mRNA in the 30 to 74*S* region of the gradient even after a 2-hr period of exposure to actinomycin D. A very limited fraction of mRNA in the 30 to 74*S* region may, in fact, combine with a 40*S* ribosome subunit and serve as a precursor to polyribosomes, although no clear-cut experimental demonstration of this fact presently exists [*see* discussion by Spirin (16)].

Some properties of the 30 to 74*S* RNA are as follows. (i) The nucleotide composition and sedimentation velocity of RNA isolated from the 30 to 74*S* region are the same as for RNA isolated from polyribosomes. (ii) The RNA is associated with protein, and this complex is heterogeneous as evidenced by its distribution between 30 and 74*S*. (iii) The quantity of RNA in the 30 to 74*S* region does not change when infected cultures are exposed to actinomycin D. This demonstrates that no significant fraction of this material serves as a precursor for polyribosome formation. (iv) After exposure to actinomycin D, the quantity of viral mRNA in the 30 to 74*S* region remains unchanged. Such cells, however, are unable to incorporate amino acids into proteins. This suggests that this mRNA is in a nonfunctional state, although alternative explanations have not been excluded. Many of the properties that we have observed for 30 to 74*S* RNA are in close agreement with Spirin's (16) findings for the RNA of informosomes in embryos of the loach.

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