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Transcriptional Complexity of Vaccinia Virus In Vivo and In Vitro

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The transcriptional complexity of vaccinia virus both in vivo and in vitro has been measured by using DNA:RNA hybridization with RNA in excess. In vivo, "early" or prereplicative RNA was found to saturate at 25% or one-half of the viral genome. "Late" or postreplicative RNA from infected HeLa cells saturated at 52% or essentially the entire genome. This well-regulated transcriptional pattern of the virus in vivo was not maintained in vitro. In a number of experiments a range of saturation values from 40 to 50% was obtained for in vitro synthesized RNA. The complexity of polyadenylated and non-polyadenylated RNA, as well as total purified 8 to 12S RNA released from the virus, was indistinguishable from purified high-molecular-weight virion-associated RNA with a sedimentation value of >20S and equivalent to total in vitro synthesized RNA. No additional hybrid formation was observed in experiments in which total in vitro RNA and late in vivo RNA from infected HeLa cells were combined, suggesting that the virus does not transcribe in vitro DNA sequences that are not also transcribed during productive infection. Approximately 15% complementary RNA was detected when radiolabeled total in vitro RNA was allowed to reanneal with late in vivo RNA, while as much as 8% of the in vitro synthesized RNA was found to be complementary.

The biogenesis of functional mRNA molecules in eucaryotic systems is a subject of intensive investigation in a number of laboratories and is a prominent topic in current molecular biology (for recent reviews, see references 5, 6, 10-12, 23, 24, 33, 39). Because of the complex nature of eucaryotic systems, progress has been slow and limited to specialized mRNA's (see reviews above) or to cells productively infected or transformed by DNA viruses (2, 9, 15, 25, 32, 34-38).

Recently, we have reported the synthesis and characterization of a high-molecular-weight virion-associated RNA by vaccinia virus in vitro (30, 31). The ability to chase such pulse-labeled, virion-associated high-molecular-weight RNA into 8 to 12S RNA components that were subsequently extruded from the virus suggested a precursor-product relationship. Other experiments showed that very small amounts of high-molecular-weight RNA were polyadenylated, whereas much larger amounts of the lower-molecular-weight RNA were polyadenylated upon cleavage. Methylation of the cleavage fragments was also suggested. Moreover, nucleic acid hybridization-competition studies indicated that considerable sequence homology existed between these two size classes of in vitro synthesized vaccinia RNA.

The present communication reports the results of a series of experiments designed to quantitate the extent of homology between the high-molecular-weight virion-associated RNA and the released 8 to 12S mRNA. If the proposed hypothesis of the biogenesis of vaccinia mRNA from a high-molecular-weight precursor (30) is biologically significant, this quantitative information is a prerequisite for understanding the system and in designing future experiments to test the validity of the hypothesis.

MATERIALS AND METHODS

Cells and virus. Suspension cultures of HeLa cells were concentrated to 1.0×10^7/ml and inoculated with 10 PFU of vaccinia virus (strain WR) per cell. After a 15-min adsorption period at 37°C, the infected cells were diluted to 5×10^5/ml in culture medium in either the presence or absence of 20 μg of cytosine arabinoside per ml. Mock-infected cells were treated in a similar fashion. After 2 h in the presence of cytosine arabinoside or 8 h in its absence, the cells were harvested by centrifugation, suspended in 10 mM Tris-hydrochloride (pH 7.4)-10 mM NaCl-1.5 mM Mg^{2+}, and allowed to swell in ice water. The cells were disrupted with a Dounce homogenizer, and nuclei and debris were removed by centrifugation. RNA was extracted from the cytoplasmic fraction essentially as described by Oda and Joklik (29), digested with RNase-free DNase, extracted with phenol, and passed through a column of

Sephadex G-50. The RNA in the void volume was concentrated by ethanol precipitation.

Synthesis and purification of vaccinia virus RNA. Vaccinia virus RNA was synthesized in vitro in a reaction mixture containing 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, 0.2 mM UTP, and purified virus at a concentration of 1.6 to 3.1 absorbancy units at 260 nm per ml. After incubation at 37°C for 40 min, Na_3EDTA was added to a final concentration of 20 mM. A portion was removed for the isolation of total RNA, made 0.5% with sodium dodecyl sulfate (SDS), and concentrated by ethanol precipitation overnight at −20°C after the addition of NaCl to 0.2 M. Viral particles were sedimented from the remainder of the reaction volume by centrifugation at 38,000 × *g* for 30 min and suspended by sonic treatment into 20 mM Na_3EDTA. Virion-associated high-molecular-weight RNA was isolated by bringing the virion suspension to 0.5% with SDS, followed by the addition of NaCl to 0.2 M. RNA was then precipitated with ethanol and held overnight at −20°C. Virion-released 8 to 12S RNA was obtained by adjusting the virus-free supernatant to 0.5% SDS–0.2 M NaCl and precipitation with 2 volumes of absolute ethanol overnight at −20°C. The above precipitates were collected by centrifugation, suspended in appropriate volumes of 10 mM sodium acetate (pH 5.1)–10 mM Na_3EDTA containing 0.5% SDS, and extracted three times by using the hot phenol-SDS method as described by Girard (17). The final aqueous layer was made 0.2 M with NaCl and precipitated with ethanol. The precipitates were collected by centrifugation, dissolved in 10 mM Tris-hydrochloride (pH 7.4)–0.1 M NaCl–1 mM Na_3EDTA containing 0.1% SDS, and subjected to two cycles of precipitation from 2 M LiCl at 4°C for 16 to 18 h as described by Baltimore and Girard (3). The precipitates were then dissolved in 10 mM Tris-hydrochloride (pH 8.4)–0.1 M NaCl containing 0.1% SDS and precipitated by ethanol at −20°C. The virion-associated and virion-released RNAs were prepared for a preliminary fractionation on SDS-sucrose gradients by dissolving in 0.1 volume of 10 mM Tris-hydrochloride (pH 8.4)–0.1 M NaCl–0.1% SDS to which 0.9 volume of 10 mM Tris-hydrochloride (pH 8.4)–0.1% SDS was added. The samples were boiled for 20 s and rapidly quenched in ice water prior to layering onto 36-ml gradients of 15 to 30% sucrose in 10 mM sodium acetate (pH 5.1)–0.1 M NaCl–0.1% SDS. After centrifugation for 18 h at 20,000 rpm in a Spinco SW27 rotor at 25°C, the gradients were fractionated into 1-ml portions from the bottom, and the UV absorption profile was determined at 260 nm. Appropriately sedimenting RNA fractions were individually pooled and precipitated by the addition of 2 volumes of absolute ethanol at −20°C. The precipitated RNA was collected by centrifugation and dissolved at a concentration of less than 100 μg/ml in 0.1 volume of 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl followed by the stepwise addition of 0.9 volume of Me_2SO. The samples were then heated at 60°C for 2 min, diluted with 4 volumes of distilled water, and precipitated with ethanol after the addition of NaCl to 0.2 M. The precipitates were collected by centrifugation, dissolved in 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl containing 0.1% SDS, and boiled for 20 s followed by rapid quenching in ice water before fractionation on a second series of SDS-sucrose gradients. The profile of RNA sedimentation was determined by absorption at 260 nm. Appropriate fractions were pooled and ethanol-precipitated three times from 10 mM Tris-hydrochloride (pH 8.4)–0.2 M NaCl. The total virion, the purified virion-associated high-molecular-weight, and the virion-released 8 to 12S RNA fractions were dissolved in 50 mM Tris-hydrochloride (pH 7.4)–2 mM $MgCl_2$ and digested at 37°C for 60 min with RNase-free DNase at a concentration of 60 μg/ml. The samples were then made 0.1% with SDS and extracted once with an equal volume of 88% phenol at room temperature. The aqueous layers were made 0.2 M with NaCl and precipitated with ethanol. All RNA fractions were passed through Sephadex G-50 columns, and the RNA in the void volume was collected for use in the hybridization studies.

The purified 8 to 12S RNA was further fractionated into polyadenylated [poly(A)+] and non-polyadenylated [poly(A)−] moieties by selection on polyuridylic acid [poly(U)]-Sepharose columns as described by Adesnik et al. (1).

Hybridization experiments using RNA synthesized in vitro. The DNA used in these experiments has been characterized in detail by Grady and Paoletti (18a). Tritium-labeled vaccinia DNA (specific activity, 5.2×10^5 cpm/μg) in 0.01× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) was sheared at 25,000 lb/in² with a French press. Hybridization reactions contained varying amounts of unlabeled, in vitro synthesized RNA and 0.006 μg of denatured, ^{3}H-labeled vaccinia DNA in 0.5 ml of 0.12 M PB (PB = phosphate buffer containing equimolar amounts of NaH_2PO_4 and Na_2HPO_4). Each sample was layered with paraffin oil to prevent evaporation and incubated at 60°C for 22 h. At the end of the incubation, each sample was diluted into 3.0 ml of 0.12 M PB containing 0.4% sodium lauryl sulfate at 60°C, and the DNA in hybrid form was separated from the DNA that remained single stranded by passage over a column of hydroxylapatite (DNA grade HTP, Bio-Rad Laboratories) at 60°C. The hydroxylapatite chromatography procedure and the method for determining the proportion of the total counts present in hybrids have been published (18). Two or more control reactions containing only denatured, ^{3}H-labeled vaccinia DNA were always run in parallel to each set of experimental samples. The small amount of DNA-DNA self-reaction (1 to 2%) detected in the controls was subtracted from the experimental data to obtain the proportion of the vaccinia genome represented as RNA transcripts in the various RNA preparations.

Hybridization experiments with RNA from HeLa cells at various times after infection with vaccinia virus. Hybridization reactions were run and the amount of DNA in the hybrid form was assayed essentially as described for in vitro synthesized

RNA. In this instance, however, the RNA used was from HeLa cells and was extracted at various times after infection with vaccinia virus in the presence or absence of cytosine arabinoside. Experiments were also carried out with RNA from uninfected cells. Since large quantities of RNA were used in these experiments, no attempt was made to collect trichloroacetic acid precipitates of the hydroxylapatite column fractions on filters. Instead, 500 μg of calf-thymus DNA carrier was added to each fraction from the hydroxylapatite column; then trichloroacetic acid was added to 5%, and after 1 h the nucleic acid precipitate in each fraction was collected by centrifuging at 2,000 × *g* for 30 min. After the supernatants were poured off, 0.6 ml of 1.0 N HCl was added to each sample, and the samples were hydrolyzed at 80°C for 2 h. Finally, the samples were cooled and counted in 10 ml of Aquasol (New England Nuclear Corp.).

Measurement of the amount of complementary RNA. Mixtures were prepared by using 8 μg of ^{3}H-labeled vaccinia total RNA synthesized in vitro (specific activity, 2,726 cpm/μg) and either 90 μg of unlabeled in vitro RNA or 190 μg of unlabeled late infected HeLa cell RNA in 0.6 ml of 6× SSC. The mixtures were denatured in boiling water for 3 min, cooled to 74°C, and layered with paraffin oil. Samples (0.15 ml) were removed immediately and at 3, 6, and 24 h. Each sample was diluted into 2.0 ml of SSC and then split into two equal portions. One portion of each sample was treated with 20 μg of RNase (Worthington Biochemicals Corp.) per ml for 30 min at 37°C. The other portion was used as a control. At the end of the incubation period, 25 μg of calf-thymus DNA carrier was added to each portion, and the nucleic acids were precipitated with 5% trichloroacetic acid. After 1 h, the trichloroacetic acid precipitates were collected on Whatman GF/C filters. When dry, the filters were placed in glass counting vials, 0.6 ml of 1.0 N HCl was added, and the tightly capped vials were incubated at 80°C for 2 h. The vials were then cooled, and the samples were counted by using 10 ml of Aquasol. The amount of complementary RNA was taken as the percentage of the control counts that were resistant to RNase in the treated portion of the sample.

RESULTS

Purification of virion-associated high-molecular-weight RNA and virion-released 8 to 12S RNA synthesized in vitro by vaccinia virus. The presence of virion-associated high-molecular-weight RNA synthesized in vitro by vaccinia virus and its stability under stringent denaturing conditions of 99% Me_2SO have been documented (28, 31). Figure 1 shows a typical sedimentation profile of virion-associated high-molecular-weight RNA (Fig. 1A) and virion-released 8 to 12S RNA (Fig. 1B) obtained on the second set of SDS-sucrose gradients as described above. It should be noted that the sedimentation profile of virion-associated high-molecular-weight RNA is broader than that of the released 8 to 12S RNA. Further fractionation of either RNA size class on aqueous or denaturing gradients did not reveal any major deviations from the sedimentation profiles shown in Fig. 1 (31; unpublished data). For the hybridization studies reported here, sections of the gradients equivalent to I, II, I + II, and III were recovered and used as purified virion-associated high-molecular-weight and purified virion-released 8 to 12S RNA, respectively. It should be noted that species of RNA sedimenting at a rate greater than 12S are also included in the cut of 8 to 12S RNA, but the latter designation will be used throughout for convenience.

DNA:RNA hybridization of purified virion-associated high-molecular-weight RNA, purified virion-released 8 to 12S RNA, and total RNA synthesized by vaccinia virus in vitro. The results of DNA:RNA hybridization, using excess RNA derived from purified virion-associated high-molecular-weight RNA, purified virion-released 8 to 12S RNA, and total RNA synthesized by vaccinia virus in vitro, are shown in Fig. 2. Figure 2A shows that no difference was observed among the three RNA preparations in either the kinetics of hybridization or the final plateau level attained. Similar results were obtained with three independently prepared sets of in vitro RNA. On a fourth occasion, total and purified high-molecular-weight

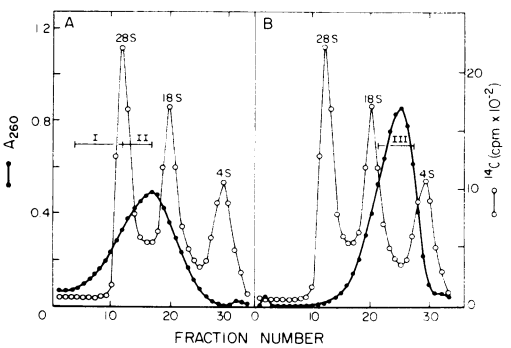

FIG. 1. *Sedimentation profile of virion-associated high-molecular-weight RNA and virion-released 8 to 12S RNA synthesized by vaccinia virus in vitro. The sedimentation profile of virion-associated high-molecular-weight RNA (A: ●) and virion-released 8 to 12S RNA (B: ●) synthesized by vaccinia virus in vitro obtained on the second set of SDS-sucrose gradients as described in the text is shown. The sedimentation profile of [^{14}C]uridine-labeled HeLa cell rRNA (○) was obtained from a parallel gradient. RNAs sedimenting in the regions denoted by I, II, and III were collected and used as the more rapidly sedimenting fraction (I) or more slowly sedimenting fraction (II) of virion-associated high-molecular-weight RNA (I + II) or purified virion-released 8 to 12S RNA (III) and used in the DNA:RNA hybridization studies. A_{260}, Absorbance at 260 nm.*

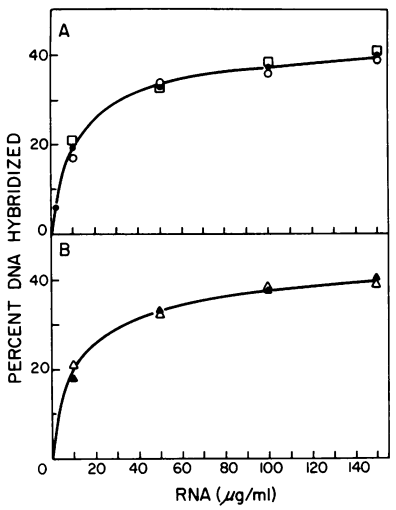

FIG. 2. *DNA:RNA hybridization using in vitro synthesized vaccinia virus RNA. (A) shows the kinetics and extent of hybridization of total RNA* (●), *purified virion-associated high-molecular-weight RNA* (□), *and purified virion-released 8 to 12S RNA synthesized in vitro by vaccinia virus* (○). *(B) plots the kinetics and extent of hybridization obtained with purified poly(A) + 8 to 12S RNA* (△) *and poly(A) − 8 to 12S RNA* (▲) *synthesized and released by vaccinia virus in vitro.*

virion-associated RNA reached the plateau first, whereas purified virion-released 8 to 12S RNA approached the plateau more gradually (data not presented). The range of saturation values obtained for total in vitro synthesized vaccinia RNA from six different preparations ranged from 40 to 50%, suggesting some degree of variability for in vitro RNA synthesis.

The possibility existed that the complexity of virion-released 8 to 12S RNA was artificially high due to the presence in this fraction of degraded fragments or abortive transcripts from the high-molecular-weight RNA. Since virion-associated high-molecular-weight RNA is essentially non-polyadenylated (30), whereas mature RNA molecules are polyadenylated, a comparison of the hybridization properties of the poly(A)+ and poly(A)− RNA obtained from the 8 to 12S fraction should clarify this situation. Figure 2B shows that the kinetics and extent of hybridization of poly(A)-containing virion-released 8 to 12S mRNA are indistinguishable from the RNA fraction deficient in poly(A) content, as defined by the ability to be bound to poly(U)-Sepharose under our experimental conditions.

Furthermore, these subfractions of purified virion-released 8 to 12S RNA are indistinguishable in the rate and extent of hybridization from the purified total virion-released 8 to 12S RNA and purified virion-associated high-molecular-weight RNA, as well as total viral RNA synthesized in vitro (compare Fig. 2A and B). The identity of poly(A)+ and poly(A)− vaccinia RNA confirms previous findings as reported by Nevins and Joklik (27). The origin of poly(A)− molecules may be due to the cleavage of the RNA fragments from high-molecular-weight precursors under conditions in which ATP is limiting (30; unpublished data).

DNA:RNA hybridization of subfractions of the purified virion-associated high-molecular-weight RNA. Since the purified virion-associated high-molecular-weight RNA demonstrated a greater degree of sedimentation heterogeneity than did the purified virion-released 8 to 12S RNA (Fig. 1), subfractions of this RNA were obtained to determine whether differences in complexity could be demonstrated. As Fig. 3 indicates, the more rapidly sedimenting virion-associated RNA (Fig. 1A, pool I) is indistinguishable in either kinetics of hybridization or complexity from the less rapidly sedimenting fraction of this RNA (Fig. 1A, pool II). Thus, by these criteria the bulk of purified virion-associated high-molecular-weight RNA cannot be subfractionated into different complexities on the basis of the sedimentation rate.

An important feature to be noted from Fig. 3 is that purified virion-associated high-molecular-weight RNA exhibits the most rapid reassociation kinetics observed (compare kinetics with an equivalent RNA fraction shown in Fig. 2). As shown in Fig. 3, saturation is essentially obtained at 15 μg of RNA per ml, whereas as shown in Fig. 2, significantly greater concentrations of RNA are required to achieve saturation. The rapid reassociation kinetics seen in Fig. 3 suggest that all of the RNA sequences transcribed are present at rather equivalent concentrations. Other preparations of total in vitro synthesized RNA as well as purified virion-associated high-molecular-weight RNA and purified virion-released 8 to 12S RNA showed a slower approach to the final plateau, although the final saturation level was approximately the same. In the latter cases the difference is probably due to a portion of the genome being transcribed with significantly lower efficiency than the bulk of the genome in the situation depicted in Fig. 3. Thus, at least two variables exist in the in vitro reaction: (i) the difference in total RNA transcription (40 to 50%) and (ii) the relative abundances of various RNA transcripts. The causes for these observed variations are not known, but may reflect subtle differences in the quality of different preparations of purified virus.

DNA:RNA hybridization with excess RNA

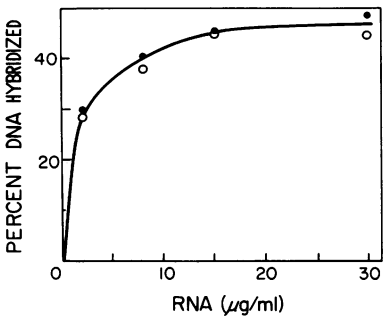

FIG. 3. *DNA:RNA hybridization of subfractions of the purified virion-associated high-molecular-weight RNA synthesized by vaccinia virus in vitro. The rate and extent of hybridization of the more rapidly sedimenting virion-associated RNA (●; Fig. 1A, pool I) and the less rapidly sedimenting portion of the virion-associated RNA (○; Fig. 1A, pool II) are shown.*

derived from vaccinia virus-infected HeLa cells. Previous reports on the transcriptional complexity of vaccinia virus in vitro (20, 27) presented saturation values much lower than those obtained in these experiments. In view of this, it was felt necessary to investigate the transcriptional complexity of vaccinia virus in vivo.

In productive vaccinia infection a facile temporal transition is marked by viral DNA replication. Events occurring prior to and not dependent upon viral DNA replication are considered "early" or prereplicative. For example, viral mRNA transcribed in the presence of cytosine arabinoside, an inhibitor of DNA synthesis, is considered early or prereplicative RNA, whereas those RNA sequences not transcribed prior to and dependent upon DNA synthesis or whose appearance is prevented by cytosine arabinoside are considered "late" or postreplicative (29).

The reassociation kinetics of early or prereplicative RNA obtained from HeLa cells at 2 h after infection with vaccinia virus in the presence of cytosine arabinoside are shown in Fig. 4A. Early RNA from infected HeLa cells saturated at 25% or one half of the viral genome. RNA purified from uninfected HeLa cells shows no reaction with purified vaccinia DNA. Late viral RNA, extracted at 8 h postinfection in the absence of the drug, saturated at 52% or the entire genome (Fig. 4B).

Figure 4B also shows that neither total in vitro viral RNA preparation that independently saturated at 40 or 50% showed any additive effect with late RNA from infected HeLa cells. Thus, although vaccinia virus is not restricted to the transcription of early sequences in vitro, the in vitro system does not transcribe vaccinia DNA sequences that are not also transcribed at late times during productive infection; i.e., no nonsense transcripts are made in vitro.

Presence of complementary RNA in vivo and in vitro. The presence of complementary RNA sequences synthesized by vaccinia virus both in vitro and in vivo has been reported (7, 8, 13). These observations are confirmed by our studies for when one of the in vivo and one of the in vitro viral RNA preparations were examined, complementary RNA was observed in each (Table 1). There was approximately 15% complementary RNA detected when radiolabeled total in vitro RNA was allowed to react with late infected HeLa cell RNA. Perhaps as much as 8% of the in vitro synthesized total

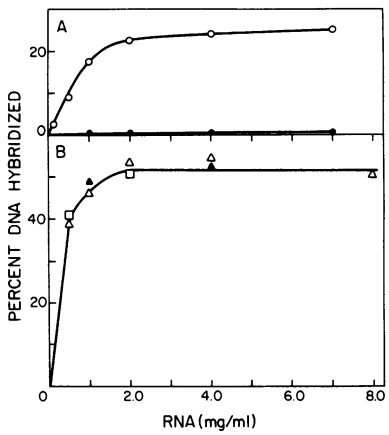

FIG. 4. *DNA:RNA hybridization with excess RNA derived from vaccinia virus-infected HeLa cells. The hybridization of early or prereplicative RNA synthesized by vaccinia virus in infected HeLa cells at 2 h postinfection in the presence of cytosine arabinoside is shown in (A: ○). Failure to form hybrids with vaccinia virus DNA by the use of RNA from uninfected HeLa cells is also shown in (A: ●). (B) shows the hybridization kinetics observed with late or postreplicative RNA synthesized by vaccinia virus in infected HeLa cells at 8 h postinfection in the absence of cytosine arabinoside (△). Also shown in (B) is the extent of hybrid formation when the following RNA fractions were added: (▲) 4 mg of late infected in vivo RNA per ml and 100 μg of total in vitro synthesized RNA per ml or 1 mg of late infected in vivo RNA per ml and 25 μg of total in vitro synthesized RNA per ml (the total in vitro synthesized RNA was found to saturate at 50% in other experiments); (□) 2 mg of late in vivo RNA per ml and 50 μg of total in vitro synthesized RNA per ml or 0.5 mg of late in vivo RNA per ml and 12 μg of total in vitro synthesized RNA per ml (the total in vitro synthesized RNA used in this latter instance independently saturated at 40%).*

TABLE 1. *Amount of complementary RNA present in total RNA synthesized in vitro and in late infected cell RNA*[a]

Time of incubation (h)	Labeled RNA resistant to RNase (%)	
	In vitro	Late in vivo
0	2.0	1.4
3	2.0	15.0
6	2.7	16.3
24	8.1	15.2

[a] Mixtures of RNA were prepared by using 8 μg of ^{3}H-labeled vaccinia virus total RNA synthesized in vitro (specific activity, 2,726 cpm/μg) and either 90 μg of unlabeled total in vitro RNA or 190 μg of unlabeled late in vivo RNA. Hybrid formation was detected as described in the text.

RNA is also complementary. The origin of this complementary RNA during transcription was not determined.

DISCUSSION

The extent of transcription by vaccinia virus both in vivo and in vitro has been determined. In vivo approximately one-half of the viral genome is transcribed early. This estimate is in excellent agreement with that obtained by Oda and Joklik (29) by hybridization competition and somewhat higher than that reported by Kaverin et al. (22). All of the genome transcripts are found at late times, and again our results are in good agreement with published values (29).

The extent of transcription by vaccinia virus in vitro via its endogenous DNA-dependent RNA polymerase (21, 26) was shown to be much more extensive than previously reported (20, 27). The reason for this variation is not clear. Possible reasons include the following: (i) different viral variants used, (ii) use of purified viral cores versus purified whole virions as used in our studies, or (iii) technical aspects of the assay. With respect to the last point it is noteworthy that all previous determinations were done with DNA immobilized on filters. Evidence has been reported that under certain conditions there is a preferential loss of hybridized DNA from the filters (19). The hybridization method used in our studies avoids this problem. However, one possible difficulty with the use of hydroxylapatite is that DNA fragments that are partly hybridized and partly single stranded will bind to the column and be scored as being completely in hybrid form. If enough of these partly hybridized fragments occur, they could cause the final saturation level to be overestimated. This possibility was ruled out by showing that results identical to those obtained with hydroxylapatite are also obtained when hybridization is measured by use of the N1 endoribonuclease from *Neurospora crassa* (data not presented). It should also be noted that the saturation values obtained cannot be attributed to residual DNA present in our RNA preparations. No hybrid formation was detected after the RNA samples were subjected to alkaline hydrolysis, reconstituted to appropriate buffer and ionic strength, and subjected directly to hybridization conditions.

There appear to be two types of variation in the in vitro RNA synthesis reactions. The total extent of transcription was found to vary from 40 to 50%, and from differences in the reassociation kinetics observed (see text) a variability in the frequency of transcription of certain regions of the genome was also suggested. The reason for these variabilities in the in vitro reaction is not immediately clear. Nevertheless, our studies show that the transcriptional complexity of vaccinia RNA is not regulated in vitro as it is in vivo. Essentially all of the viral genome can be transcribed in the in vitro reaction. However, since no additive effects of in vitro RNA were detected with late in vivo RNA, it appears that the virus in vitro does not transcribe DNA sequences that are not also transcribed in vivo. In vitro, therefore, the virus does make biologically relevant transcripts from essentially its entire genome.

Since the endogenous DNA-dependent RNA polymerase of vaccinia virus is able to transcribe late sequences in vitro, it would not appear necessary that the synthesis of a new RNA polymerase be invoked for "specific" in vivo transcription of late sequences. More likely, rigid temporal control is maintained in vivo by de novo synthesis of a protein functional in maintaining a regulatory control on transcription or by retaining in vivo a strict conformational integrity of either the polymerase itself or a conformational relationship between the polymerase and its DNA template. These regulatory mechanisms might not be available in the in vitro reaction.

Recently we have reported the synthesis and characterization of a virion-associated high-molecular-weight RNA synthesized by vaccinia virus in vitro (30, 31). A series of experiments showed that this large RNA could be cleaved into smaller 8 to 12S RNA fragments by an endogenous viral RNase. Some of these 8 to 12S fragments were then polyadenylated and released from the viral core. Sequence homology was observed by hybridization competition between the 8 to 12S released RNA and the virion-associated high-molecular-weight RNA. We

suggested that one possible interpretation of the data was that RNA processing, i.e., the biogenesis of functional mRNA from larger RNA precursors, was a functional mechanism in the vaccinia system. Although other explanations of the data, such as dysfunctional termination of transcription, have still not been ruled out, the data reported here are supportive of an RNA processing hypothesis. The transcriptional complexity of purified virion-released 8 to 12S RNA was shown to be indistinguishable from the complexity residing in purified virion-associated high-molecular-weight as well as total in vitro synthesized RNA. The data are consistent with the hypothesis that 8 to 12S poly(A)+ mRNA molecules are derived from virion-associated high-molecular-weight precursors and that essentially all of the informational complexity is conserved. Since high-molecular-weight RNA is not released from the virus (30, 31), a precursor-product relationship is strongly suggested.

Until similar studies are performed in infected cells, however, one must defer the interpretation that RNA processing by vaccinia virus is biologically significant.

We are aware of the potential hazards involved in working with RNA due to aggregation. We have shown that the high-molecular-weight virion-associated RNA remains large even under stringent denaturing conditions of Me_2SO (28, 31). The possibility of contamination of purified 8 to 12S virion-released RNA by degraded fragments or aborted nascent chains of unrelated high-molecular-weight RNA has been eliminated by the selection of poly(A)+ 8 to 12S RNA that must be then considered as "mature" mRNA. It is not as readily possible to exclude contamination of virion-associated high-molecular-weight RNA by 8 to 12S components. The following points, however, bear on this problem: (i) the purified 8 to 12S poly(A)+ mRNA has all of the complexity of total in vitro synthesized RNA; (ii) no aggregation of the 8 to 12S RNA is observed on refractionation on SDS-sucrose gradients as described above; and (iii) no extensive segregation of 8 to 12S or other lower-molecular-weight species is observed when the purified virion-associated high-molecular-weight RNA is resedimented on SDS-sucrose gradients. Since at one point both types of RNA are denatured (see Materials and Methods), one would have to invoke an unknown factor(s) to cause the complete aggregation of one RNA preparation and no aggregation of the other. A more reasonable interpretation is that this virion-associated RNA is indeed large.

It should be noted that no significant sequence complexity unique to the high-molecular-weight RNA was detected. Such large transcripts would appear to be required for the synthesis of high-molecular-weight polypeptides. It is possible that these sequences comprise specific regions of the genome not efficiently transcribed and were, therefore, not measured by our techniques. Alternately, in the cases in which we have compared the various classes of RNA synthesized in vitro in terms of complexity, as much as 10% of the transcribable vaccinia genome was not represented (see above). This portion represented as much as 1.2×10^7 daltons of information, sufficient to encode a good number of large polypeptides.

If one assumes that poly(A)-containing mRNA molecules purified from the released 8 to 12S RNA fraction are functional, mature mRNA molecules, then the following estimates of informational complexity can be made. When 1.2×10^8 daltons is used as the genomic complexity of the vaccinia genome (4, 14, 16, 18a), at a complexity level of 40% transcription in vitro, one would expect approximately 150 to 200 distinct messages of 8 to 12S that might then be generated from the cleavage of approximately 30 to 50 high-molecular-weight precursor molecules (30, 31).

Although one may not be able to physically separate the two strands of the vaccinia genome, our results suggest that over any particular stretch of DNA nucleotide sequences, the transcriptionally active strand can be separated from its transcriptionally silent complement. This will be useful in ordering the biological expression of the vaccinia genome.

ACKNOWLEDGMENTS

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