# Analysis of Vaccinia Virus Transcriptional Complexity In Vitro and In Vivo: Characterization of RNase $T_1$-Resistant 5'-Terminal Oligonucleotides

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Vaccinia virus mRNAs synthesized in vitro and in vivo, polyadenylated leader sequences synthesized in vitro in the absence of added GTP, CTP, or UTP or in the presence of 20 μg of actinomycin D per ml, and high-molecular-weight RNA synthesized in vitro under limiting ATP concentrations were labeled specifically in the cap structure using $[\alpha\text{-}^{32}P]GTP$ and vaccinia-soluble enzyme extracts. The complexity of RNase $T_1$-resistant 5'-terminal oligonucleotides was analyzed by two-dimensional polyacrylamide gel electrophoresis. Approximately 190 unique $T_1$-resistant 5'-terminal oligonucleotides were observed from vaccinia virus 8 to 12S RNA synthesized in vitro. A somewhat greater complexity was observed with polyadenylated leader sequences and actinomycin D RNAs where unique $T_1$-resistant oligonucleotides ranged from approximately 210 to 280 5'-terminal fragments. On a composite fingerprint of the above RNAs, more than 300 identifiable unique $T_1$-resistant 5'-terminal oligonucleotides were observed. Significantly, close to 300 $T_1$-resistant fragments were derived from RNA sedimenting faster than 18S on denaturing sucrose gradients. Analysis of vaccinia RNAs synthesized in vivo in the absence of either de novo protein synthesis or DNA replication or in the presence of actinomycin D gave essentially similar profiles of 5'-terminal $T_1$-resistant oligonucleotide fingerprints consisting of approximately 200 fragments. Analysis of the 5'-terminal $T_1$-resistant oligonucleotides of vaccinia RNAs present after DNA replication showed essentially the same pattern of early $T_1$-fragments albeit in reduced amounts but in addition revealed a complex pattern of $T_1$-resistant oligonucleotides unique to this class of vaccinia RNA.

Vaccinia virus is a large cytoplasmic DNA virus with a genome of approximately $1.2 \times 10^8$ daltons. The transcriptional strategy of the virus in vivo has been demonstrated to be tightly controlled temporally; hence, three phases of transcription have been identified: (i) an "immediate early" phase, defined as the transcription of the genome in the absence of de novo protein synthesis during which the viral genome is still in a coated form; (ii) an "early" phase of transcription, defined temporally as the expression of the genome in the absence of DNA replication; and (iii) a "late" phase of transcription, which covers the expression of the genome after DNA replication. These stages of temporal regulation of transcription have been quantified, and there is general agreement that early transcriptional complexity involves 50% of the transcribable sequences, whereas 100% of the transcribable sequences is present at late times (3, 25,

28). Although quantitative but not qualitative differences in protein synthesis can be distinguished between immediate early and early (6), significant discrepancies in RNA sequences present at these two stages of viral replication have been reported (3, 20, 21).

The presence of an endogenous DNA-dependent RNA polymerase in poxviruses (20, 23) has allowed the study of transcription of vaccinia in vitro. The complexity of the sequences found during in vitro vaccinia transcription is identical to that present at late times in vivo (3, 28). Calculations based on the informational content of vaccinia have been made from which it is concluded that approximately 150 to 200 polypeptides of average molecular weights can be colinearly coded by the vaccinia genome (3, 28).

The in vitro transcripts of vaccinia virus are RNAs of approximately 1,000 bases and contain polyadenylate sequences at the 3' end and are

capped and methylated at the 5' end. In addition, two other types of RNAs have been detected in vitro. One type consists of small RNAs ranging in size from 20 to several hundred bases and include the leading sequence of the transcript. They are capped and methylated at the 5' end and polyadenylated at the 3' end (polyadenylated leader sequences [PALS]). These RNAs have been synthesized in vitro in reactions where the addition of UTP, CTP, or GTP is omitted from the RNA polymerase reaction, or in the presence of actinomycin D, or from virus treated with UV light (14, 31). The simplest explanation for the existence of these RNAs is that they are abortive transcription products, but that they may be involved in mRNA maturation has also been considered (31).

The other type of in vitro transcription product is a high-molecular-weight (HMW) RNA, which is detected in in vitro reactions containing either suboptimal concentrations of ATP or heat-treated virus (18, 26). Whether this HMW RNA represents a precursor to mature vaccinia mRNA or arises because of dysfunctional termination of the transcript under the perturbing conditions of synthesis is not clear.

Because of the transcriptional complexity present in this system, studies involving these RNAs as populations give little information in understanding the various relationships between them or the regulation of their transcription. In an attempt to decrease this complexity, we elected to study these RNAs by concentrating on the 5' ends. The unique structures of the 5' end of vaccinia RNA, $^{m}$GpppGm-- and $^{m}$GpppAm-- (35), and the presence of the pertinent enzymatic functions for generating these structures in crude soluble extracts of vaccinia cores (9), allow a very sensitive and specific labeling of the 5' ends of these RNAs (22). The specificity of $T_1$ RNase generates an oligonucleotide containing only a single non-cap guanosine moiety located at the 3' end of the digestion product. Thus, the combination of these two methodologies allow one to generate RNase $T_1$-resistant 5'-end-labeled oligonucleotides that can be readily analyzed by two-dimensional polyacrylamide gel electrophoresis. We have used this fingerprinting method to analyze the complexity, relationship, and regulation of various classes of vaccinia virus RNAs in vivo and in vitro.

Analysis of the $T_1$-resistant 5'-end oligonucleotides derived from PALS by two-dimensional polyacrylamide gel electrophoresis revealed very complex but distinct fingerprints containing both unique and apparently shared oligonucleotides (data not shown). Interestingly, there were fewer $T_1$-resistant oligonucleotides derived from total in vitro synthesized RNA (~190 spots) than

from any individual class of PALS (~215 to 280 spots; data not shown). Because of the complexity of the $T_1$ fingerprint patterns observed with the vaccinia RNA and PALS synthesized in vitro, it was of interest to determine the number of unique $T_1$ spots resolvable from a composite analysis of the four PALS and in vitro vaccinia RNA. Thus, an equivalent number of counts per minute from each of the PALS and RNA was combined, digested with $T_1$ and analyzed by two-dimensional polyacrylamide gel electrophoresis. Figure 1A shows the radioautographic profile of such a composite digestion, whereas a tracing of the $T_1$ spots is shown in Figure 1B. Over 300 definable $T_1$ spots are observed from such a composite gel, indicating both the high resolution of this system and the complexity of the 5' ends of vaccinia transcripts derived in vitro.

In vitro, under conditions of reduced ATP concentrations during RNA synthesis, vaccinia virus synthesizes HMW RNA. This HMW RNA has been partially characterized (4, 14, 24, 26–28, 30). It is approximately four to five times larger than the mature vaccinia mRNA that is released from the viral cores. The HMW RNA is capped and methylated at the 5' end, but is not significantly polyadenylated (27). The HMW RNA can be cleaved while still virion-associated in the presence of ATP and released from the virus or cleaved by an endoribonuclease activity present in soluble extracts of vaccinia cores (13, 27, 29). The in vitro translation products of the HMW RNA are similar to the polypeptides synthesized in in vitro translation systems programmed with mature monocistronic-sized mRNA, and the translated regions of the HMW RNA are thought to be at or near the 5' end of the RNA (4). The pattern of the 5' ends derived by $T_1$-digestion of HMW RNA (Fig. 2) is as complex or more complex than any individual PALS or total 8 to 12S RNA and approaches the complexity of the composite analysis of PALS and RNA. Approximately 300 distinct $T_1$ fragments can be derived from HMW RNA and the overall $T_1$ fingerprint obtained with HMW RNA is distinguishable from those obtained with individual PALS or in vitro monocistronic RNA.

The number of 5'-terminal $T_1$-resistant oligonucleotides present in the HMW RNA suggests that the synthesis of HMW RNA can be initiated from any vaccinia promotor and not from a unique subset of promotors. It should be pointed out, however, that the multitude of termini may not represent HMW RNA transcription initiation but rather a display of post-transcriptional modifications. It is assumed in our study that the ribonucleoside triphosphate initiating transcription is the base present as the penultimate base in the capped RNA. The roles, if any, of the
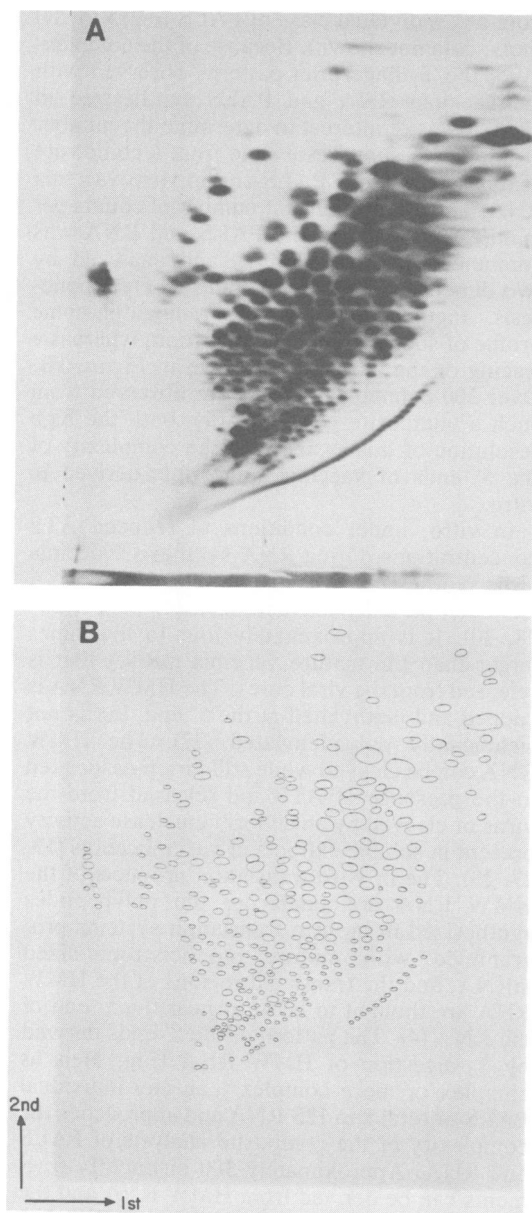
ride (pH 8.4); 10 mM magnesium acetate; 10 mM dithiothreitol, 0.05% Nonidet P-40; ATP, CTP, GTP, and UTP at 2 mM each; purified vaccinia virus at 4 U (absorbance at 260 nm) per ml; and 10 $\mu$M $S$-adenosyl homocystine to minimize synthesis of capped RNAs. The reactions were supplemented with additional ATP every 30 min and terminated after 2 h by the addition of sodium dodecyl sulfate to 0.1% and $Na_3EDTA$ to 10 mM. Reactions were extracted with phenol, unincorporated nucleotides were removed by sieving on Sephadex G-50 resin, and polyadenylate RNA was selected by chromatography on polyuridylate-Sepharose columns as previously described (26, 30). PALS were synthesized in similar reactions, except that either UTP, GTP, or CTP was omitted from the reactions for the synthesis of ACG, ACU, or AGU PALS, respectively. Actinomycin D PALS were synthesized in the presence of all four triphosphates in reactions containing 20 $\mu$g of actinomycin D per ml. PALS and RNA were labeled directly in the cap structures in in vitro reactions containing 50 mM Tris-hydrochloride (pH 7.8), 2.5 mM magnesium acetate, 1 mM dithiothreitol, 10 $\mu$M $S$-adenosyl methionine, $[\alpha\text{-}^{32}P]GTP$ (2,000 to 3,000 Ci/mmol; Amersham Corp.), RNA at 100 $\mu$g/ml, and soluble vaccinia core extracts as the source of the capping enzymes (9). After 30 min at 37°C, the reactions were made 0.1% with sodium dodecyl sulfate and 10 mM with $Na_3EDTA$, extracted with phenol, desalted on Sephadex G-50, and ethanol precipitated in preparation for digestion with $T_1$ RNase. Analysis of the methylation products indicated that the methyl label was incorporated exclusively in the cap structure and that methylation was greater than 90% complete. Results comparable to those reported here with these labeling strategies have also been obtained in other control experiments using internally labeled RNAs with subsequent selection of the 5'-terminal oligonucleotides by boric acid gel chromatography or from tritium methyl-labeled RNAs followed by fluorographic detection (data not presented). Carrier yeast tRNA was added to labeled vaccinia RNA so as to contain 40 $\mu$g total nucleic acid in 10 $\mu$l of 10 mM Tris-hydrochloride–1 mM $Na_3EDTA$ (pH 7.0), boiled for 30 s, followed by rapid quenching in ice water before the addition of 4 $\mu$g of $T_1$ RNase (Calbiochem). The samples were digested at 37°C for 30 min and then dried in vacuo. Control experiments indicated that the above conditions were sufficient for complete digestion. The dried samples were prepared for electrophoresis by resuspending in 2 volumes of 9 M urea and 1 volume of glycerol-dye mixture (50% glycerol, 8 mg each of bromophenol blue and xylene cyanol$_{FF}$ per ml, and 20 mg of trypan red per ml), final volume 10 $\mu$l. The samples were boiled and rapidly quenched immediately before application to the first-dimension polyacrylamide gel. The two-dimensional gel system was essentially that of deWachter and Fiers (8). The first-dimension gel measured 37.5 cm by 7.5 cm by 0.75 mm and was composed of 14% acrylamide–0.48% $N,N'$-methylene-bis-acrylamide containing 7 M urea. A saturated solution of citric acid was added to a final pH of 3.3, and catalysts were added as follows: 0.001% $FeSO_4\cdot7H_2O$, 0.038% ascorbic acid, and 0.01% $H_2O_2$. Electrophoresis was at room temperature at 400 V for approximately 20 h with 0.025 M citric acid buffer. When the trypan red dye had migrated 23 cm, gel strips (1.3 cm wide and 30.2 cm from the origin) were

FIG. 1. Composite RNase $T_1$ fingerprint of ACG, AGU, ACU, and actinomycin D PALS and vaccinia RNA synthesized in vitro. Approximately $1 \times 10^5$ cpm from each of the ACG, AGU, ACU, and actinomycin D PALS and vaccinia virus RNA synthesized in vitro were pooled and digested with $T_1$ RNase, and the products were analyzed by two-dimensional polyacrylamide gel electrophoresis in the directions indicated. (A) Radioautograph of a 3-day exposure of the gel, (B) schematic representation of the more than 310 distinct $T_1$ spots enumerated on the composite radioautograph. Vaccinia virus RNA was synthesized in vitro in reactions containing 50 mM Tris-hydrochlo-

excised and soaked for 5 min in 0.4 M Tris-borate (pH 8.2) containing 7 M urea. The second-dimension gel measured 35 cm by 43 cm by 0.75 mm and was composed of 23% acrylamide, 1.5% N,N'-methylene-bis-acrylamide, 7 M urea, and 0.05 M Tris-borate (pH 8.2). (NH$_4$)$_2$S$_2$O$_8$ (0.027%) and N,N,N',N'-tetramethylethylenediamine (0.06%) were added, and the gel was allowed to polymerize while immersed in a water tank. Electrophoresis was performed at room temperature with 0.05 M Tris-borate (pH 8.2) at 600 V for approximately 18 h until the bromophenol blue dye marker had migrated 24 cm from the center of the first-dimension gel strip. Autoradiography of the wet gels was performed at −70°C by using Kodak XR-2 film and Dupont Cronex intensifying screens.

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vaccinia-associated endoribonuclease activity that cleaves HMW RNA (29) and a 5'-polynucleotide kinase activity capable of modifying termi-

ni internally derived from the cleavage of HMW RNA for subsequent capping reactions (32) must still be considered.

It is interesting to note that the PALS and HMW RNA contain a greater complexity of 5'-terminal T$_1$-resistant oligonucleotides than the in vitro 8 to 12S RNA. Since both PALS and HMW RNA are derived from reactions where the concentration of at least one triphosphate is limiting, the number of 5' ends may represent heterogeneity of initiation of the RNA transcript under these nonoptimal conditions. Heterogeneity at the 5' ends of mRNAs has been detected in vivo for other animal viruses such as adenovirus, simian virus 40, and polyoma (1, 5, 10, 11, 15, 17). Heterogeneity at the 5' ends may arise either throughout a stretch of DNA around the initiation site or more localized through the



FIG. 2. RNase T$_1$ fingerprint of HMW RNA synthesized by vaccinia virus in vitro. HMW RNA was synthesized by vaccinia virus in vitro under conditions of limiting ATP concentrations. The RNA was extracted and sedimented on denaturing sucrose gradients (27, 30). RNA sedimenting faster than HeLa 18S ribosomal RNA was recovered from the dimethylsulfoxide-sucrose gradients, labeled specifically in the 5'-terminal cap structure, and digested with RNase T$_1$ as described in Fig. 1. A 3-day exposure of a gel on which $5 \times 10^5$ cpm were fractionated in the dimensions indicated is shown.
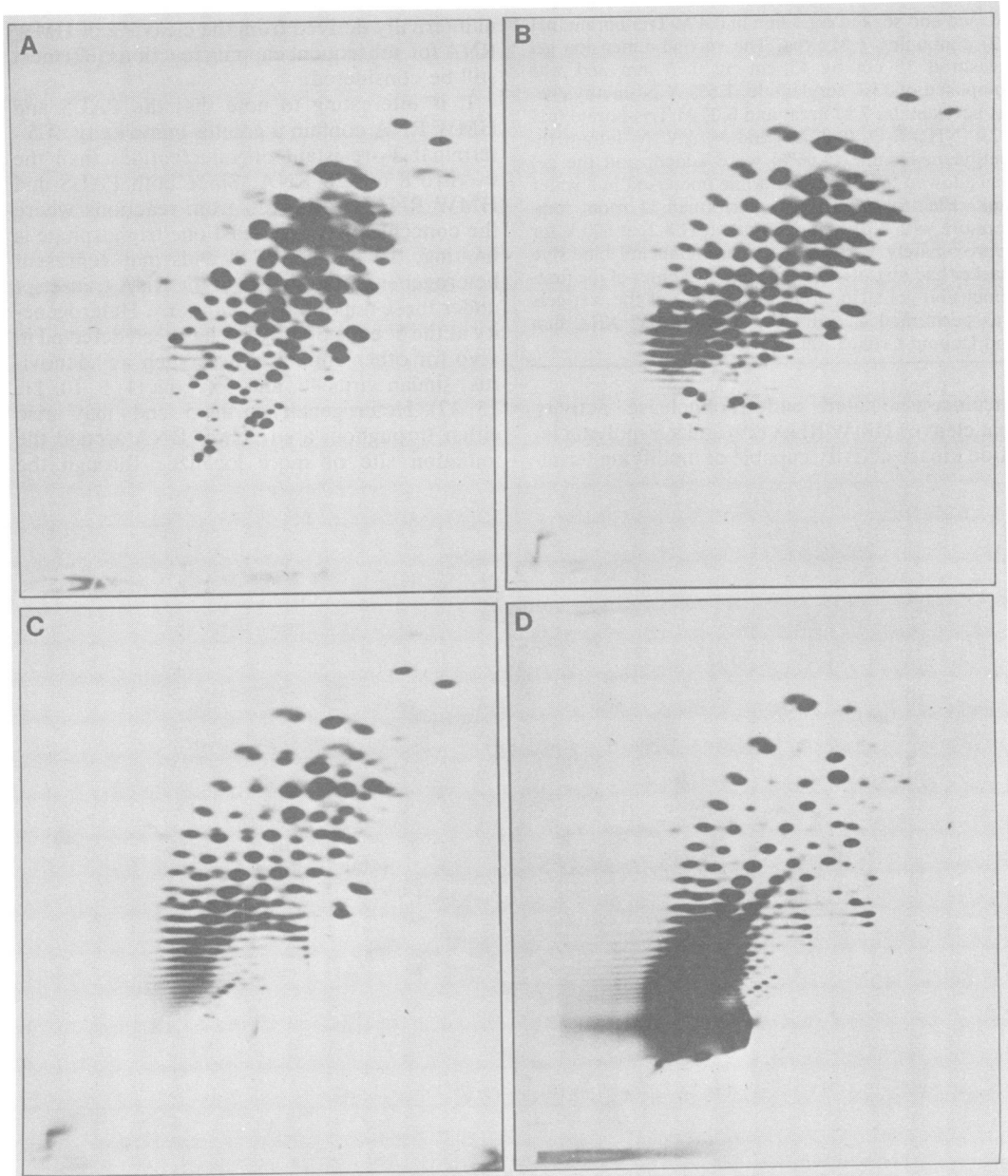
FIG. 3. RNase $T_1$ fingerprints of in vitro and in vivo vaccinia virus RNAs. To prepare in vivo RNAs, 1-liter volumes of HeLa cells at $4 \times 10^5$ to $5 \times 10^5$ cells per ml were infected with 100 PFU of vaccinia virus per cell. After a 15-min absorption period, the infected cells were diluted into medium containing 100 $\mu$g of cycloheximide per ml to inhibit de novo protein synthesis for the analysis of immediate early RNA, 40 $\mu$g of cytosine arabinoside per ml to prevent viral DNA replication for the analysis of early RNA or without drugs, for the analysis of total late RNA. Cells infected in the absence of drugs were harvested at 6 h, whereas those containing the inhibitors were harvested at 3 h postinfection. Infected cells were dounce-homogenized to rupture the cytoplasms, and nuclei and debris were removed by low-speed centrifugation. The cytoplasmic preparations were treated with 2% Sarkosyl NL-97, and RNA was purified through CsCl cushions as described (16). Polyadenylate RNA was selected by chromatography on poly(U)-Sepharose columns, and viral sequences selected by hybridization to vaccinia DNA as described below. Purified vaccinia virus was prepared essentially as described by Joklik (19). Virions were lysed at a concentration of 50 U (absorbance at 260 nm) per ml in 10 mM Tris-hydrochloride (pH 7.8)–50 mM $\beta$-mercaptoethanol–100 mM NaCl–10 mM $Na_3$EDTA–1% Sarkosyl NL-97–26% sucrose. Proteinase K was added to a concentration of 100 $\mu$g/ml, and the lysate was incubated at 37°C overnight. DNA was extracted by addition of an equal volume of phenol-chloroform (1:1). The organic phase was

choice of a purine initiating the transcript generating either GpppA- or GpppG-capped RNA. In view of the latter, it should be pointed out that approximately 95% of the caps present in the ACU PALS are GpppA-type caps (31; unpublished data). It is significant that if the polymerase makes a choice as to purine initiation, that it is predominantly a rightward shift in base selection since a leftward shift would generate a nonmethylated G residue adjacent to the penultimate base in the capped RNA. This G residue would be sensitive to RNase $T_1$ digestion, generating a significant population of GpppAmGp products. This is not evident on any of the fingerprints. Further, it should be pointed out that the numbers of $T_1$-resistant spots derived from the 5′ ends by this type of analysis are minimal, since oligonucleotides of identical base composition but different sequence might not be resolved. In this regard, direct sequence analysis of the $T_1$ fragments recovered from gels indicate considerable heterogeneity, suggesting that the $T_1$ spots represent multiple species (unpublished data).

To understand the temporal regulation of vaccinia transcription in vivo, the $T_1$-resistant 5′-terminal oligonucleotides derived from in vivo vaccinia RNA were analyzed and compared with the fingerprint obtained with in vitro RNA (Fig. 3A).

When the $T_1$-resistant 5′-terminal oligonucleotides derived from immediate early vaccinia RNA (Fig. 3B) were compared with those derived from early transcripts (Fig. 3C), essentially similar fingerprints were obtained, although some unique $T_1$ fragments peculiar to each temporal class of RNA were detected. The similarity in the fingerprints of these two classes of vaccinia RNA is not indicative of major differences in sequence complexity, consistent with the data reported by Boone and Moss (3).

Analysis of the $T_1$-resistant 5′-terminal oligonucleotides derived from late vaccinia transcription is shown in Fig. 3D. It should be noted that this RNA is not selected for unique late transcription products and therefore contains RNA sequences synthesized early but still present after DNA synthesis. There is a clear difference in the fingerprint of these late sequences compared with the early sequences. Most of the $T_1$-resistant 5′-terminal oligonucleotides detected in early RNA are still present at late times, although in reduced concentrations. Significantly, however, additional $T_1$-resistant 5′-terminal oligonucleotides are detected at late times with a clear transition in distribution as detected by two-dimensional polyacrylamide gel electrophoresis.

The reason for the lack of resolution of these apparently large 5′-terminal oligonucleotides derived from RNA synthesized in the absence of metabolic inhibitors is not known, but the possible charge heterogeneity in the capped moieties of the RNAs may be a contributing factor (2).

removed, and the aqueous phase reextracted until the interphase was clear. Two additional extractions with chloroform were performed, and the aqueous phase dialyzed extensively against 10 mM Tris-hydrochloride (pH 7.4) containing 0.1 mM $Na_3EDTA$ at 4°C. DNA was concentrated to approximately 100 μg/ml with Ficoll. Aminobenzyloxymethyl paper was obtained from Schleicher and Schuell and converted to the diazotized form by the specifications of the manufacturer. Vaccinia DNA was transferred to diazobenzyloxymethyl (DBM) paper as follows. Purified DNA (1.7 mg) in 25 ml was randomly cleaved by sonication, mixed with an equal volume of 2% agarose, poured into a block (8 by 14 by 0.4 cm) and allowed to solidify. The DNA-containing agarose was then treated successively with 0.25 M HCl, 0.5 M NaOH, and 1 M sodium acetate buffer (pH 4.0), and transferred to a sheet of DBM paper (8 by 14 cm) essentially as described by Wahl et al. (34). The DBM paper was cut into strips (2 by 14 cm) and prehybridized for 6 h at 42°C in 50% formamide, 5× SSC (0.15 M NaCl and 0.015 M sodium-citrate), 5× Denhardt reagent (0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, and 0.02% Ficoll) (7); 1% glycine; and 500 μg of sonicated, denatured salmon sperm DNA in 50 mM sodium-phosphate buffer (pH 6.5). Polyadenylate RNA (200 to 500 μg) isolated from vaccinia-infected HeLa cells was dissolved in 3 ml of hybridization buffer (50% formamide, 5× SSC, 1× Denhardt, 0.1 mM $Na_3EDTA$, 0.2% sodium dodecyl sulfate, 100 μg sonicated, denatured salmon sperm DNA, and 10% dextran sulfate in 20 mM sodium-phosphate buffer [pH 6.5]) (34), heated for 65°C for 30 min, then hybridized to the DBM paper in a siliconized glass vial at 42°C for 20 h. The DBM paper was washed five times with 40 ml of 2× SSC and 0.2% sodium dodecyl sulfate at room temperature for 15 min per wash, three times with 0.2× SSC, and 0.2% at 60°C for 30 min per wash, and finally two times in 10 ml of 2 mM $Na_3EDTA$ (pH 8.0). The RNA was eluted from the DNA by boiling the DBM paper in 3 ml of deionized, redistilled, autoclaved water for 3 min followed by rapid quenching in a dry ice-methanol bath. Sodium-acetate (pH 5.5) was added to 0.2 M and the RNA recovered by ethanol precipitation. In control experiments, a second round of hybridization selection of radiolabeled RNA gave an identical $T_1$ fingerprint suggesting no significant levels of host RNA contamination. In vivo RNAs were first treated with sodium-periodate and analine to remove the endogenous blocking guanosine residue as described (12) before labeling in reactions identical to those used for in vitro RNAs described in the legend to Fig. 1. In vitro RNA was fractionated on sucrose gradients, and the polyadenylate 8 to 12S RNA was selected as described above. Radioautographs of in vitro RNA (5 × 10⁵ cpm, 3-day exposure), immediate early, early, and late RNA (each at approximately 1.5 × 10⁵ cpm per gel, 5-day exposure) are shown in panels A, B, C and D, respectively.

The in vitro 8 to 12S mRNA does not exhibit these $T_1$ oligonucleotides peculiar to the late class of in vivo RNA even though sequence complexity is identical (3, 28) indicating that methylation patterns in the cap (2) or perhaps other additional modifications occur only in vivo. Alternatively, it may simply reflect relative abundancies of different messages (3).

The analysis of the 5'-terminal $T_1$-resistant oligonucleotides from early and late vaccinia RNAs raises some interesting questions. More than 200 unique 5'-terminal oligonucleotides are present at both early times. This is close to the expected number of messages derived from a colinear transcription of the entire genome. What is the contribution of heterogeneous termini derived from in vivo synthesis? Are late genes transcriptionally silent at early times, or is there some attenuation phenomenon which would allow additional $T_1$-resistant 5'-terminal fragments from attenuated late gene expression to be detected at early time?

If heterogeneity at the 5' ends is minimal, then the total number of vaccinia transcripts would be higher than expected from a colinear flow of information from DNA to RNA. Such a process might occur if overlapping genes were present or if significant information was transcribed from both strands of DNA (33).

The display of 5'-terminal $T_1$-resistant fragments derived from late RNA does not allow an estimate of numbers because of the smearing obtained on gels. The simplest interpretation is that these $T_1$ fragments are derived from late messages; however, it might also be considered that the fragments represent peculiar post-transcriptional 5'-terminal modifications of early RNAs at late times making them poor messages during late stages of viral replication.

It will be interesting to decipher some of these questions on the mechanism of vaccinia transcription. The complexity of the $T_1$-resistant 5'-terminal oligonucleotides obtained from any individual class of vaccinia RNA is so great as to make meaningful direct comparisons between fingerprints quite difficult. The complexity of the vaccinia system is much too great to resolve in any significant way the transcriptional events and regulatory processes occurring in vitro and in vivo by studying populations of RNAs. To reduce the complexity and decipher the transcriptional strategy involved in vaccinia gene expression and regulation, it will be necessary to utilize the tools of cloning and focus attention on manageable regions of the genome. Such approaches have already been reported (36) and are also being pursued in our laboratory.

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