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# In Vitro Transcription of the Inverted Terminal Repetition of the Vaccinia Virus Genome: Correspondence of Initiation and Cap Sites

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Specific RNAs synthesized in vitro by vaccinia virus cores were analyzed with the aid of DNA from the terminal 9,000 base pairs of the genome that was cloned in phage λ, pBR322, and the single-stranded phage f1. Three mRNA's coding for polypeptides with molecular weights of 7,500 (7.5K), 19K, and 42K were shown to have sizes and map positions similar to those described for mRNA's made early in infection. A previously undescribed transcript made in vivo and in vitro, with a 5′ end at about 8.7 kilobase pairs from the end of the genome, was also detected. After chemical removal of the terminal 7-methylguanosine residue, the 5′ ends of the RNAs were specifically labeled by enzymatic capping and then mapped by gel electrophoresis of nuclease-resistant RNA·DNA hybrids, as well as by hybridization of the end-labeled RNA to immobilized DNA restriction fragments. Analysis of the purified cap structures demonstrated that three of the mRNA's have both $m^7G(5')pppA^m$ and $m^7G(5')pppG^m$ ends, indicating some degree of terminal heterogeneity. The fourth transcript has exclusively $m^7G(5')pppA^m$ ends. By synthesizing RNA in the presence of [$\beta$-$^{32}$P]GTP, it could be shown that cap sites correspond to sites of initiation of RNA synthesis.

Vaccinia virus contains a linear double-stranded DNA genome with a molecular weight of approximately $120 \times 10^6$, the expression of which is temporally regulated (18). At least initially, early RNA is synthesized, capped, and polyadenylylated by enzymes packaged within the virus core (13, 14, 21, 31). Indeed, the cell-free translation products of mRNA synthesized in vitro by vaccinia virus cores are very similar to those directed by early mRNA from infected cells (9, 25). Nevertheless, the sequence complexity of in vitro RNA more closely resembles that of late RNA, which is approximately one strand-equivalent of the genome, than early RNA, which is only half of one strand-equivalent (7, 23). This discrepancy probably results from the in vitro synthesis of very small amounts of RNA from late sequences detectable only by hybridization carried out in vast RNA excess (7). More detailed studies on the fidelity of the in vitro system, however, had to await the development of techniques capable of analyzing individual mRNA species.

Recently, three mRNA's made early during vaccinia virus infections were shown to be encoded within the 10,000-base-pair (bp) inverted terminal repetition of the vaccinia virus genome (10, 32, 33). Analysis of individual mRNA's was facilitated by the cloning of a 9,000-bp terminal DNA fragment in coliphage λ. To determine whether the same mRNA's are made in vitro, the previously cloned DNA segment, as well as new subclones in plasmid and single-stranded DNA vectors, were used. The results indicate that mRNA's of similar lengths, map positions, and coding capacities are synthesized in vivo and in vitro. Furthermore, the incorporation of [$\beta$-$^{32}$P]GTP into the 5′ ends of the three mRNA's signifies that initiation of synthesis occurs at the cap sites. In addition, the ends of these RNAs made in vitro and in vivo contain both $m^7G(5')pppA^m$ and $m^7G(5')pppG^m$, suggesting terminal heterogeneity of the type found with simian virus 40 and polyoma virus mRNA's (8, 12).

## MATERIALS AND METHODS

**Construction of recombinant plasmids and single-stranded DNA phage.** All DNA recombinant work was done in accordance with the current National Institutes of Health guidelines. A recombinant λ phage designated λA7/1, containing the terminal *Eco*RI fragment of vaccinia virus (WR strain) DNA, was described previously (32). The recombinant DNA was subcloned by cleavage with *Sal*I and insertion of the fragments into the unique *Sal*I site of the plasmid pBR322. This DNA was then used to transform *Escherichia coli* HB101, and recombinant colonies were identified by their resistance to ampicillin, sensitivity to tetracycline, and by colony hybridization (27), using labeled vaccinia virus DNA as a probe. Two clones

described below and designated pAG1 and pAG4 were used in this study. Growth of cells, plasmid amplification, cell lysis, and plasmid purification were essentially as described elsewhere (6).

The entire 9,000-bp vaccinia virus DNA insert from λA7/1 was inserted within the unique *Eco*RI site of the replicative form of f1 phage R199, kindly provided by G. F. Vovis (5), and propagated in *E. coli* K528, an $F^+$ *tra* $D^-$ strain. Recombinant f1 were detected by plaque hybridization, using $^{32}P$-labeled vaccinia virus DNA as a probe. Of seven independent recombinant plaques that contained phage with full-length inserts, all were in the same orientation. One of these, designated f1 SV1, was used for this study. Growth and purification of the recombinant phage and preparation of single-stranded and replicative-form phage DNA were as described elsewhere (5).

**Synthesis and purification of RNA.** Immediate early RNA was obtained from the cytoplasm of HeLa cells infected with vaccinia virus in the presence of cycloheximide and purified by centrifugation through CsCl as previously described (9). In vitro RNA was synthesized with purified vaccinia virus particles. For the synthesis of unlabeled RNA, the reactions contained 50 mM Tris-hydrochloride (pH 8.5); 0.05% Nonidet P-40 detergent; 10 mM dithiothreitol; 5 mM ATP; 1 mM each of GTP, CTP, and UTP; 10 mM $MgCl_2$; and 100 μM *S*-adenosylmethionine (AdoMet). The reactions were started by addition of 1 to 10 U (absorbance at 260 nm) of purified vaccinia virus per ml. After 15 min at 37°C, fresh AdoMet was added to a concentration of 100 μM, and the reaction was continued for 15 more min. Typically, 10 to 20 μg of RNA was synthesized per ml. To prepare unmethylated RNA, 50 μM *S*-adenosylhomocysteine (AdoHcy) was used in place of AdoMet. Uniformly labeled RNA was prepared in a similar manner, using 200 μM [α-$^{32}P$]-UTP (3,000 to 30,000 cpm/pmol). Labeling with [β-$^{32}P$]GTP was performed in 50-μl transcription reactions with 200 μM [β-$^{32}P$]GTP (800,000 cpm/pmol).

All reactions were terminated by adding EDTA to a final concentration of 10 mM and placing the tubes on ice. Virus particles were removed by centrifugation at 15,000 × *g* for 5 min. In most cases, only the released RNA present in the supernatant was used. Sodium dodecyl sulfate was added to a concentration of 0.25%, and the mixture was extracted twice with equal volumes of phenol-chloroform (1:1). After adjusting the aqueous phase to 0.2 M NaCl, the nucleic acids were precipitated with 2.5 volumes of ethanol at −20°C or in a dry ice-ethanol bath. The ethanol precipitation step was repeated, and the pellets were dissolved in 3.5 ml of water. To this was added 3.5 g of CsCl, and the solution was layered on a 0.5-ml cushion of 5.7 M CsCl–50 mM EDTA in a polyallomer tube. The RNA was pelleted and separated from any DNA by centrifugation at 40,000 rpm in an SW50.1 rotor for 18 to 20 h at 18°C. The RNA pellets were dissolved in 0.05 M Tris-hydrochloride (pH 8.5)–0.2 M NaCl. After several cycles of ethanol precipitation, the RNA was stored in small portions at −70°C. When purifying RNA from small reaction volumes, 20 μg of rabbit liver tRNA per ml was added as carrier after phenol extraction.

Polyadenylylated RNAs were purified by oligodeoxythymidylic acid-cellulose chromatography essentially as described elsewhere (1).

**In vitro cap labeling of RNA.** RNA made in vivo and either methylated or unmethylated RNA synthesized in vitro were treated with periodate and β-eliminated to remove the 5′-terminal guanosine residue as described previously (20). After three cycles of ethanol precipitation, the RNA pellets were dissolved in water and stored at −70°C. Approximately 100 μg of β-eliminated RNA was incubated in 0.1-ml reactions containing 50 mM Tris-hydrochloride (pH 7.5), 2 mM dithiothreitol, 2.5 mM $MgCl_2$, 250 μCi of [α-$^{32}P$]GTP (300 to 400 Ci/mmol), and 20 μl of RNA guanylyltransferase purified by DEAE-cellulose and DNA cellulose chromatography (28). After 30 min at 37°C, 20 μl of fresh enzyme was added. Except where indicated, the reaction also contained 50 μM AdoMet, which was added again at 20 min after the start of the reaction. Where specified, AdoMet was omitted, and 50 μM AdoHcy was present. Reactions were terminated by the addition of EDTA to 10 mM and sodium dodecyl sulfate to 0.2%, and RNA was extracted with phenol-chloroform (1:1), and ethanol precipitated as described above. RNA was then dissolved in 0.2 ml of 50 mM ammonium acetate and filtered through a column (1 by 15 cm) of G-50 Sephadex to remove residual GTP and any small oligonucleotides. The capped RNA was recovered by ethanol precipitation and dissolved in 20 mM Tris-hydrochloride (pH 7.5).

**Nuclease S1 analysis.** The procedure of Berk and Sharp (3) was modified in several respects, the most important of which was the use of uniformly labeled (16) or cap-labeled RNA and unlabeled DNA. Hybridizations were carried out in 30 μl of 80% formamide–0.4 M NaCl–0.04 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.4]–1 mM EDTA. Either 0.1 pmol of RNA labeled with [α-$^{32}P$]UTP (3,000 cpm/pmol) or 0.1 pmol of $^{32}P$-cap-labeled RNA (80,000 cpm/pmol) and 0.2 pmol equivalent of the specific vaccinia DNA fragment was present in each reaction. The mixtures were denatured by heating at 68°C for 5 min, and hybridization occurred at 42°C for 16 to 24 h. The latter was terminated by dilution with 10 volumes of 0.25 M NaCl–0.03 M sodium acetate (pH 4.5)–1 mM $ZnSO_4$–5% glycerol containing 375 U of nuclease S1 (Miles Laboratories, Inc., Elkhart, Ind.) per ml. After incubation at 42°C for 60 min, the samples were cooled to 25°C and digested with 25 ng of RNase A (preheated to remove any DNase) for 10 to 15 min. The latter digestion was terminated by addition of sodium dodecyl sulfate to 0.25%. After two phenol-chloroform (1:1) extractions, the nucleic acids in the aqueous phase were precipitated with ethanol. The trimmed RNA·DNA hybrids were analyzed by electrophoresis in 1.8% neutral agarose gels (33), which were then vacuum dried on DEAE paper and fluorographed.

**Hybridization of RNA to immobilized DNA.** DNA restriction fragments were separated by electrophoresis on 1.5% neutral agarose gels and transferred by blotting to pretreated nitrocellulose filters or diazotized paper as described elsewhere (30). Controlled partial alkaline hydrolysis (11) was used to fragment labeled RNA before hybridization. The latter was carried out in sealed plastic bags containing 5× SSC (1× SSC is 0.15 M sodium chloride–0.015 M sodium citrate) immersed in a rocking 60°C water bath for 24 to 36 h. Unhybridized RNA was removed by repeated

washes with 2× SSC at 60°C and treatment with 10 μg of RNase A per ml in 2× SSC for 15 min at 25°C. After RNase treatment, the filters were washed three times with 25 ml of 2× SSC containing 0.5% sodium dodecyl sulfate and three times with 2× SSC. The dried filters were placed in contact with X-ray film with or without the use of intensifying screens.

**Cap analysis.** In vitro cap-labeled RNAs hybridized to DNA restriction fragments immobilized on either diazotized paper or nitrocellulose filters were localized by fluorography. Strips containing the hybridized RNAs were cut out and eluted with 0.4 ml of 90% formamide–0.04 M sodium PIPES (pH 6.4) at 65°C. After addition of 5 μg of yeast tRNA carrier, the RNA was precipitated in 70% ethanol–0.2 M NaCl. The precipitates were washed with 66% ethanol, dried, dissolved, and digested with nuclease P1 and calf alkaline phosphatase as described (29). The products were analyzed by high-voltage electrophoresis on DEAE paper at pH 3.5. Fluorographs were prepared and scanned by soft-laser densitometry (LKB Instruments, Inc., Rockville, Md.). The area under each peak was determined using a Numonics Graphic calculator.

**Hybridization selection and cell-free translation of mRNA.** Approximately 40 μg of recombinant DNA that was cleaved with the appropriate restriction enzyme to release the cloned insert was denatured by boiling in 0.2 M NaOH for 15 min, diluted with 40 ml of cold water, neutralized, adjusted to contain 5× SSC, and collected on 2.5-cm-diameter nitrocellulose membranes. Recombinant single-stranded DNA (5 μg) in 5× SSC was bound directly to nitrocellulose filters. Selection of RNA by hybridization, cell-free translation in message-dependent reticulocyte lysates, and analysis of the [$^{35}$S]methionine-labeled products by polyacrylamide gel electrophoresis were essentially as described previously (10).

**Materials.** Radioactive isotopes were purchased from Amersham Corp.; restriction enzymes were purchased from Bethesda Research Laboratories; nucleases P1 and S1 were from Yamasa Shoyu Co. and Miles Laboratories, Inc., respectively; and diazotized paper and nitrocellulose filters were purchased from Schleicher & Schuell Co.

## RESULTS

**Subcloning of DNA within the inverted terminal repetition.** Previous investigations demonstrated that early during infection, three mRNA's encoding polypeptides with molecular weights of 7,500 (7.5K), 19K, and 42K are transcribed from the 10,000-bp inverted terminal repetition of the vaccinia virus genome. The map locations of these mRNA's and cleavage sites for *Hin*cII, *Sal*I, and *Hpa*II are shown in Fig. 1. The latter studies were carried out with a 9,000-bp terminal *Eco*RI fragment of vaccinia virus DNA that was cloned in coliphage λ. To facilitate further studies, *Sal*I fragments of the recombinant λ DNA were subcloned in the plasmid pBR322. One of the plasmid recombinants used here, designated pAG4, contains *Sal*I fragment C1, which encodes early mRNA for the 7.5K polypeptide (Fig. 1). The other plasmid recombinant, pAG1, contains the *Sal*I A fragment, as well as about 1 kilobase pair (kbp) of adjacent phage λ DNA. This segment of vaccinia virus DNA encodes early mRNA's for both the 19 and 42K polypeptides (Fig. 1). The *Sal*I restriction fragments of the two plasmid recombinants alongside an *Sal*I digest of the parent phage λ recombinant DNA are shown in Fig. 2A. The 0.95-kbp fragment of pAG4 and the 4.7-kbp fragment of pAG1 comigrate with *Sal*I fragments of the λ recombinant. The 4.36-kbp bands common to both pAG4 and pAG1 represent plasmid DNA. The identities of the subcloned vaccinia DNA fragments were further established by comparison of the electrophoretic mobilities of combined *Sal*I and *Eco*RI digests of the plasmid and λ recombinants (data not shown) and by nick translating the recombinant plasmids and probing blots of *Eco*RI and *Sal*I double digests of the phage λ recombinant (data not shown).

To facilitate studies requiring DNA strand separation, the entire 9,000-bp insert from the phage λ recombinant was transferred to a single-stranded DNA phage. Although we expected some recombinants with one strand of vaccinia virus DNA and some with the other, all seven independent phage isolates with full-length inserts had the strand which hybridizes to the mRNA for the 19K polypeptide. Whether this was simply due to chance or because the other orientation of the DNA is unfavorable, perhaps because of the proximity of the multiple tandem repetitions within the end of the vaccinia virus genome (34) to the origin of phage DNA replication, is not known. Electrophoretic analysis of an *Eco*RI digest of the replicative form of one of the phage f1 recombinants alongside an *Eco*RI digest of the phage λ recombinant demonstrated comigration of the 9-kbp fragments (Fig. 2B). The 4.6-kbp fragment is derived from the phage genome. The identity of the cloned fragment was confirmed by double digestion with *Eco*RI and *Hin*cII (data not shown).

**Comparison of the cell-free translation products of selected in vivo and in vitro mRNA's.** Previous experiments showed that early mRNA's selected by hybridization to the λA7/1 recombinant DNA directed the synthesis of the 7.5 and 19K major polypeptides, a less abundant polypeptide of 42K, as well as minor polypeptides between 23 and 38K in a message-dependent reticulocyte lysate (10, 32). Similar results were obtained in this study, except that the 7.5K polypeptide, which was previously found to migrate just ahead of the unlabeled

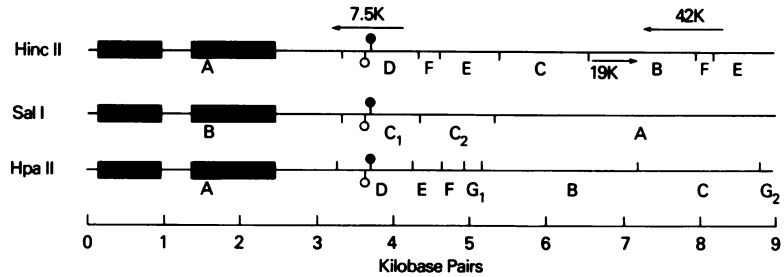


FIG. 1. *Restriction maps of the terminal fragment of vaccinia virus DNA cloned in bacteriophage lambda. Lengths are measured from the EcoRI site constructed within 50 bp of the original cross-linked end of the genome (34). The boxes enclose the two blocks of tandemly repeated 70-bp sequences previously reported (34). The symbols ♀ and ♁ refer to the DdeI and AluI sites within the HincII D fragment. Other DdeI and AluI sites within the remainder of the DNA are not shown. The arrows represent the sizes, map positions, and direction of transcription of the early mRNA's encoded within this portion of the vaccinia virus genome (33). The numbers above the arrows indicate the sizes of the polypeptides synthesized in reticulocyte cell-free systems upon addition of the specified RNA (10).*

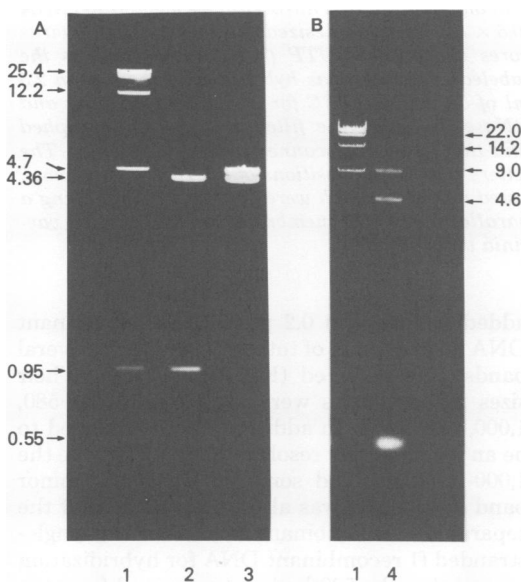


FIG. 2. *Restriction endonuclease analysis of recombinant DNAs. The following DNAs were cleaved with SalI (A) or EcoRI (B), resolved by electrophoresis on agarose gels, and stained with ethidium bromide: 1) λA7/1, 2) pAG4, 3) pAG1, and 4) f1SV1 recombinant. The sizes of the fragments (in kilobases) are indicated. The lowest band in track 4 is RNA.*

broad globin band, was now found to migrate either just behind or ahead of globin, presumably because of slight variations in protein stacking during electrophoresis (Fig. 3, λA7/1 B). Polypeptides of the same sizes were also synthesized with RNA made in vitro by vaccinia virus cores (Fig. 3, λA7/1 A).

The plasmid recombinants were used to show that the in vivo and in vitro mRNA's coding for these polypeptides have the same map locations. RNA selected by hybridization to pAG4 directed the synthesis of the 7.5K polypeptide, whereas RNA selected by hybridization to pAG1 directed the synthesis of the 42 and 19K polypeptides, as well as the 24 and 35K polypeptides (Fig. 3). Polypeptides similar in size to the latter two were previously considered to be alternative products of the mRNA's encoding the 19 and 42K polypeptides. As anticipated, synthesis of the 19K polypeptide was directed by RNA selected by hybridization to the single-stranded phage f1 recombinant DNA (Fig. 3).

**Hybridization of in vitro RNA to immobilized DNA fragments.** For a more direct analysis of RNA, [$\alpha$-$^{32}$P]UTP was used to label transcripts made in vitro by vaccinia virus cores. This RNA was then hybridized to the fragments separated by electrophoresis of a *Hinc*II digest of the phage λ recombinant, and an autoradiograph was produced. The densitometer tracing indicates that DNA sequences in fragments B, D, and E were transcribed more efficiently than sequences in A and C (Fig. 4). Similar results were obtained using $^{32}$P-labeled early RNA made in vivo (E. Barbosa, unpublished data). Slight hybridization to the A fragment is expected because the 3' end of the early mRNA encoding the 7.5K polypeptide extends beyond the *Hinc*II site as shown in Fig. 1. Although the early mRNA's for the 7.5, 19, and 42K polypeptides do not extend into the *Hinc*II C fragment, minor high-molecular weight RNAs not indicated in Fig. 1 hybridize to that region (33). Accordingly, hybridization of small amounts of labeled in vitro RNA to *Hinc*II fragment C is consistent with results of in vivo experiments.

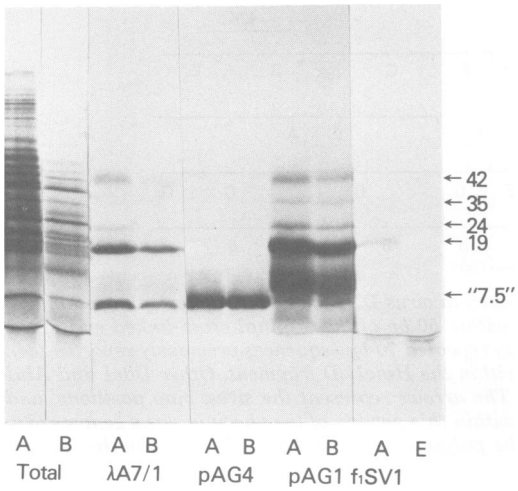


FIG. 3. *Cell-free translation products of hybridization-selected RNAs. RNA was hybridized to the indicated recombinant DNA immobilized on nitrocellulose filters and translated in a message-dependent reticulocyte lysate. The [$^{35}$S]methionine-labeled polypeptides were resolved by electrophoresis on 20% polyacrylamide gels and detected by fluorography. (A) In vitro RNA; (B) early in vivo RNA; (E) no added RNA.*

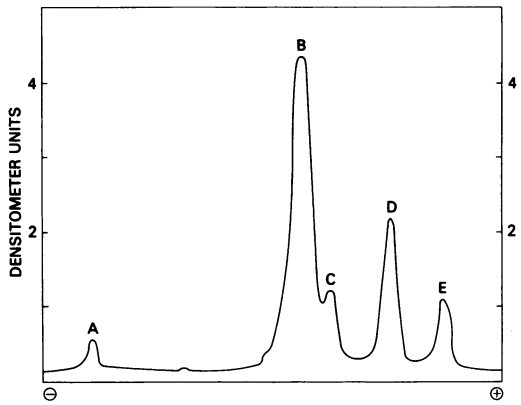


FIG. 4. *Hybridization of labeled in vitro RNA to DNA restriction fragments. Fragments produced by double digestion of λA7/1 DNA with EcoRI and HincII were separated by agarose gel electrophoresis and immobilized to a nitrocellulose membrane. RNA (0.5 × 10$^6$ cpm), synthesized in vitro by vaccinia virus cores using [α-$^{32}$P]UTP (20,000 cpm/pmol) as the labeled precursor, was hybridized to the blot in 2.5 ml of 5× SSC at 65°C for 24 h. After washing and RNase digestion, the filter was autoradiographed and the X ray was scanned with densitometer. The letters refer to the positions of the HincII fragments, listed in Fig. 1, which were determined by probing a parallel strip of the membrane with $^{32}$P-labeled vaccinia virus DNA.*

When in vitro RNA was hybridized to fragments obtained by *Eco*RI and *Hpa*II digestion of the λ recombinant, fragments B, C, and D were labeled strongly, whereas fragment G was labeled weakly; no hybridization to the *Hpa*II A fragment was detected (not shown). This result also agrees with map positions previously obtained for in vivo RNAs.

**Characterization of in vitro RNAs by nuclease S1 analysis.** In a previous study (33), we used the procedure of Berk and Sharp (3, 4) to resolve early mRNA's transcribed in vivo from the inverted terminal repetition. In that study, $^{32}$P-labeled recombinant DNA and unlabeled RNA were used to demonstrate that the mRNA for the 19K polypeptide is about 570 nucleotides long and that the mRNA's for the 7.5 and 42K polypeptides are both about 1,000 nucleotides long. The facility in labeling vaccinia virus RNA made in vitro by vaccinia virus cores and our desire to probe several different recombinant DNAs led us to use labeled RNA for this type of analysis. The species obtained by neutral agarose gel electrophoresis after nuclease S1 and RNase A digestions of [α-$^{32}$P]UMP-labeled RNA hybridized to λA7/1, pAG4, and pAG1 recombinant DNA are shown in Fig. 5A. The amounts of DNA used for hybridization were shown in control experiments to be approximately 50 times that required to saturate the added RNA. With 0.2 pmol of λ recombinant DNA and 0.1 pmol of total in vitro RNA, several bands were detected (Fig. 5A, track 1). Their sizes in base pairs were estimated to be 580, 1,000, and 2,526. In addition, there appeared to be an incompletely resolved band just above the 1,000-bp band, and sometimes another minor band of 2,430 bp was also observed. Use of the separated λ recombinant strands or the single-stranded f1 recombinant DNA for hybridization proved that the 580-bp band is derived from the light or rightward-reading strand and is therefore the mRNA for the 19K polypeptide. The other bands, including mRNA's for the 7.5 and 42K polypeptides, are derived from the heavy or leftward-reading strand (Fig. 5A, tracks 2 to 4). These results are similar to those obtained by hybridizing early in vivo RNA to $^{32}$P-labeled DNA (33).

When DNA from the recombinant plasmid pAG4, which encodes the 7.5K polypeptide, was used, a new band of 724 bp was detected in place of the 1,000-bp band (Fig. 5A, track 8). Since the mRNA for the 7.5K polypeptide extends past the *Sal*I C1 fragment (Fig. 1), only part of the mRNA was protected. The size of this truncated DNA·RNA hybrid indicates that the 3′ end of

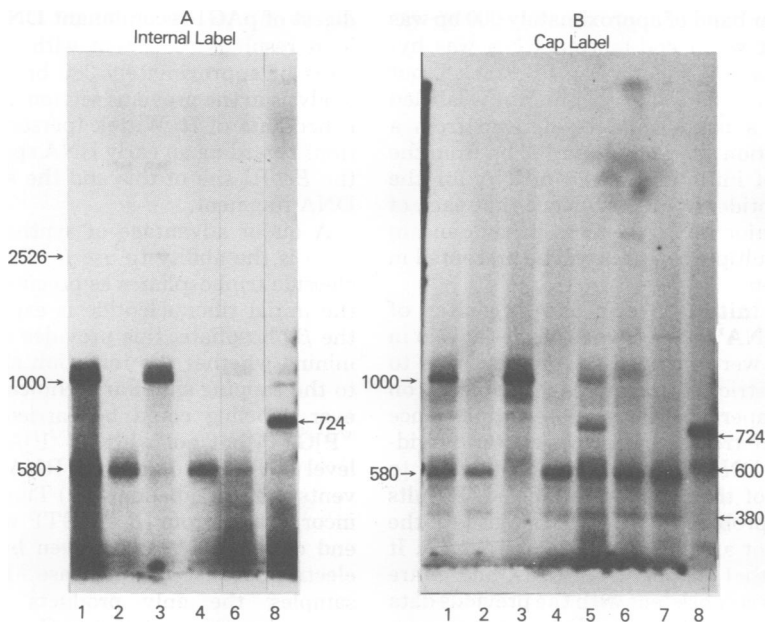


FIG. 5. *Autoradiograph of nuclease S1-resistant DNA·RNA hybrids resolved by agarose gel electrophoresis. RNA was synthesized in vitro by vaccinia virus cores using [α-$^{32}$P]UTP as the labeled precursor (internal label), or unlabeled RNA made in vitro was β-eliminated and enzymatically recapped using [α-$^{32}$P]GTP (cap labeled). The labeled RNA was then hybridized in formamide to the following unlabeled DNAs: 1) total recombinant λA7/1 digested with EcoRI, 2) λA7/1 heavy strand, 3) λA7/1 light strand, 4) single-stranded f1 recombinant DNA, 5) λA7/1 digested with EcoRI and SalI, 6) pAG1 digested with EcoRI and SalI, 7) pAG1 digested with HincII, and 8) pAG4 digested with SalI. The hybrids were digested with nuclease S1, resolved by agarose gel electrophoresis, and autoradiographed. The positions of end-labeled marker DNA fragments analyzed on the same gel are shown.*

the in vitro RNA extends about 275 bp beyond the first *SalI* site. By contrast, the pAG1 recombinant contains sequences complementary to the entire mRNA's, for the 42 and 19K polypeptides and the 1,000 and 580 nucleotide bands corresponding to these mRNA's were not truncated (Fig. 5A, track 6). Faint high-molecular-weight bands evident in the nuclease S1 analysis may represent minor species of RNA similar to those previously detected with early in vivo RNA hybridized to labeled DNA (33). In summary, nuclease S1 analysis indicated that the sizes and map positions of RNAs transcribed from the inverted terminal repetition of vaccinia virus DNA are similar in vivo and in vitro.

Nuclease S1 experiments were also carried out with cap-labeled RNA, a procedure that has not previously been described. Cap-labeling was carried out by first removing the original 5'-terminal m$^7$G residue and adding a new one enzymatically. By using [α-$^{32}$P]GTP for recapping, label was specifically introduced into the 5' end of the mRNA. This latter point was confirmed by nuclease P1 digestion and electrophoretic analysis of the products (data not shown). The nuclease S1-resistant DNA·RNA hybrids resolved in tracks 1–4, 6, and 8 of Fig. 5B were similar to those obtained with internally labeled RNA, indicating that the DNA fragments used are complementary to the 5' end, as well as the body of the message.

The detection of 1,000-, 724-, and 580-bp bands in an additional experiment in which cap-labeled RNA was hybridized to *SalI*-cleaved λ recombinant DNA was consistent with the other results (Fig. 5B, track 5). Loss of the 1,000-bp band when *Hinc*II-cleaved pAG1 DNA was used was consistent with the map positions of the mRNA for the 42K polypeptide (Fig. 5D, track 7).

A band of approximately 380 bp was detected in tracks 1, 2, 4–6, and 7 of Fig. 5B. Such a band could form by hybridization of a previously undescribed RNA species transcribed from the rightward-reading strand, starting within the *Hinc*II E fragment and extending past the end of the 9,000-bp cloned DNA segment. Additional evidence for such an RNA species will be shown

later. Another band of approximately 600 bp was most evident when end-labeled RNA was hybridized to the pAG4 clone (Fig. 5B, track 8) but could also be detected with uniformly labeled RNA. Such a band could be derived from a second initiation site more than 100 bp from the major site of initiation of the mRNA for the 7.5K polypeptide. In fact, evidence that each of the three major mRNA's made in vivo and in vitro have multiple cap sites will be presented in a later section.

**Mapping initiation and capping sites of in vitro mRNA's.** The 5′ ends of cap-labeled in vitro RNAs were mapped by hybridization to separated restriction fragments immobilized on diazotized paper or nitrocellulose filters. Since the blots were treated with RNase after hybridization, only DNA fragments complementary to the 5′ ends of the RNA were labeled. Results obtained by probing *Eco*RI/*Hpa*II digests of the λ recombinant are shown in Fig. 6, track 1A. It is apparent that fragments B, C, D, and G are labeled. This is consistent with the previous data and suggests that B, C, and D contain the cap sites of mRNA's coding for the 19, 42, and 7.5K polypeptides, respectively. Since the *Hpa*II G fragment is double molar (Fig. 1), either G1 or G2 could contain the cap site of an additional RNA. The location of that cap site within the *Hpa*II G2 fragment was demonstrated by probing a blot containing fragments from an *Hpa*II digest of pAG1 recombinant DNA (not shown). This result is consistent with the finding of a band of approximately 380 bp by nuclease S1 analysis in the previous section and with unpublished data of R. Wittek (personal communication) regarding an early RNA species that spans the *Eco*RI site of this and the adjacent cloned DNA fragment.

A major advantage of synthesizing RNA in vitro is the ability to use β-$^{32}$P-labeled ribonucleoside triphosphates as precursors. Since only the initial ribonucleotide is expected to retain the β-phosphate, this provides a way of determining whether the initiation site corresponds to the capping site. For technical reasons, however, labeling could be carried out with [β-$^{32}$P]GTP, but not with [β-$^{32}$P]ATP. (The high level of ATP required for RNA synthesis prevents efficient labeling [20].) That all of the label incorporated from [β-$^{32}$P]GTP went into the 5′ end of the RNA was proven by DEAE-paper electrophoresis of nuclease P1 digests of samples; the only products detected were $m^7G(5')pppG^m$, $m^7G(5')pppG$, and $G(5')pppG$. When the [β-$^{32}$P]GTP-labeled RNA was hybridized to the *Eco*RI/*Hpa*II fragments of the λ recombinant, bands B, C, and D were labeled (Fig. 6, track 1B). This indicated that mRNA's for the 19, 42, and 7.5K polypeptides all have $m^7G(5')pppG^m$ ends and, furthermore, that initiation of RNA synthesis occurs at the cap sites.

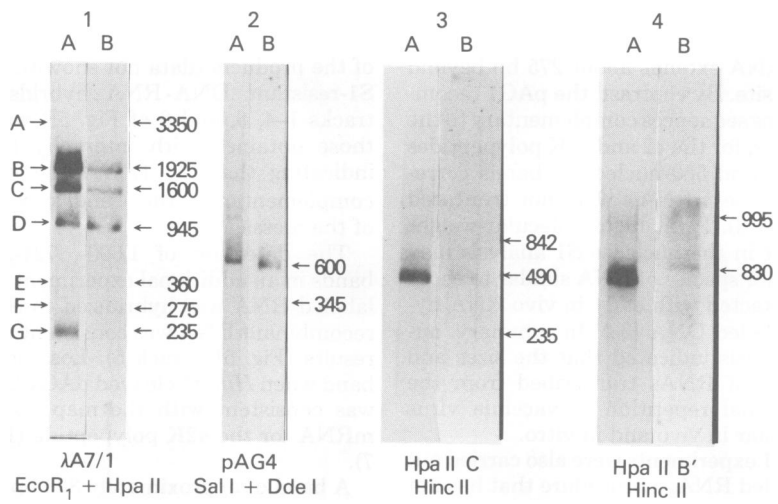


FIG. 6. *Fluorograph of cap-labeled and [β-$^{32}$P]GTP-initiated RNA hybridized to restriction fragments. Restriction fragments were resolved by agarose gel electrophoresis and transferred to diazotized paper. 1) EcoRI and HpaII digest of λA7/1 recombinant, 2) SalI and DdeI digest of pAG4 recombinant, 3) HincII digest of the HpaII C fragment, 4) HincII digest of the HpaII B fragment that had been truncated by SalI digestion. A strip of each blot was hybridized with (A) cap-labeled in vitro RNA and (B) [β-$^{32}$P]GTP-labeled RNA, as well as with $^{32}$P-labeled vaccinia virus DNA indicated by arrows. The sizes of the fragments are indicated in base pairs.*

By contrast, the *Hpa*II G fragment was not labeled.

To further demonstrate the identity of the initiation sites and the cap sites, RNA was hybridized to smaller restriction fragments. *Dde*I digestion of the *Sal*I C1 fragment cloned in pAG4 yielded a 600-bp and a 345-bp fragment (Fig. 1). When either cap-labeled or [$\beta$-$^{32}$P]GTP-labeled RNA was hybridized to these two fragments, only the larger one was labeled (Fig. 6, track 2A, B). To locate the 5′ ends of the mRNA's for the 19 and 42K polypeptides, recombinant pAG1 DNA was cleaved with *Sal*I and *Hpa*II, and the fragments were purified by agarose gel electrophoresis. One fragment referred to as *Hpa*II-B′ represents the *Hpa*II B fragment in Fig. 1 that has been slightly trimmed at the *Sal*I site; the other is equivalent to the *Hpa*II C fragment. The *Hpa*II C and B′ fragments were then cleaved with *Hinc*II, and the digests were separated by agarose gel electrophoresis and transferred to diazotized paper. Of the three fragments produced by digestion of *Hpa*II-C, only the 490-bp fragment hybridized to cap-labeled and [$\beta$-$^{32}$P]GTP-labeled RNAs (Fig. 6, tracks 3A, B). This agrees with the map position of the mRNA for the 42K polypeptide (Fig. 1). *Hinc*II digestion of *Hpa*II-B′ gives rise to two fragments, the smaller of which hybridized to cap-labeled and [$\beta$-$^{32}$P]GTP-labeled RNA (Fig. 6, tracks 4A, B). This result is consistent with the assigned map position of the mRNA for the 19K polypeptide (Fig. 1).

In summary, we obtained evidence that the mRNA's for the 7.5, 19, and 42K polypeptides are initiated with [$\beta$-$^{32}$P]GTP at their cap sites. However, the previously undescribed RNA that has a cap site within the *Hpa*II G fragment was not labeled with [$\beta$-$^{32}$P]GTP either because this RNA is initiated with ATP exclusively or is capped at an internal site.

**Evidence for heterogeneity at the 5′ ends of vaccinia virus RNAs.** When the cap-labeled RNAs hybridized to restriction fragments were eluted, digested with nuclease P1, and analyzed by electrophoresis on DEAE-cellulose paper, evidence for 5′-terminal heterogeneity was obtained. Results with RNA eluted from *Hinc*II fragments B, D, and E (Fig. 1) are summarized in Table 1. In each base, both G(5′)pppG and G(5′)pppA caps were detected. Moreover, quantitatively similar results were obtained with both in vitro and immediate early in vivo RNAs. These results suggested that more than 75% of the mRNA for the 7.5K polypeptide begins with guanosine and that the remainder begins with adenosine. Approximately the opposite ratio was obtained for the mRNA coding for the 19K polypeptide, whereas nearly equal amounts of adenosine and guanosine caps were found in the mRNA for the 42K polypeptide.

TABLE 1. *Cap analysis of in vivo and in vitro RNAs*

| *Hinc*II fragment | In vitro RNA[a] | | In vivo RNA | |
|---|---|---|---|---|
| | % G cap | % A cap | % G cap | % A cap |
| B | 25 | 75 | 26 | 74 |
| D | 76 | 24 | 80 | 20 |
| E | 57 | 43 | 48 | 52 |

[a] In vitro and in vivo RNAs were $\beta$-eliminated and enzymatically recapped with [$\alpha$-$^{32}$P]GTP. The RNA was hybridized to the indicated immobilized restriction fragments, treated with RNase A, eluted, digested with nuclease P1 and alkaline phosphatase, and analyzed by DEAE-paper electrophoresis. The percentages of G cap and A cap were determined by densitometry of fluorographs.

These results were confirmed and refined in the following manner. First, cytoplasmic RNA was purified by oligodeoxythymidylic acid-cellulose chromatography before recapping to remove minor high-molecular-weight non-polyadenylylated RNA species (33). Second, different and smaller restriction fragments were used for hybridization selection. Nevertheless, the same results were obtained for all three mRNA's (Table 2). However, now the capped fragment of the newly described RNA that hybridizes near the end of the 9,000-bp segment of DNA was separated from the mRNA for the 42K polypeptide. Moreover, that new RNA appeared to start exclusively with adenosine, explaining why we did not detect hybridization of [$\beta$-$^{32}$P]GTP-labeled RNA to the *Hinc*II G fragment (Fig. 6, tracks 1A, B).

## DISCUSSION

The presence of a full complement of enzymes necessary for synthesis and processing of mRNA within the vaccinia virus particle provides a potentially powerful tool for studying transcription. An important preliminary question, however, is whether RNAs made in vitro are similar to those made in infected cells. We analyzed RNAs encoded within the 9,000 bp of the inverted terminal repetition because this segment of the genome was the first to be cloned (32) and the early mRNA's encoded there are well characterized (10, 33). These RNAs are approximately 1,050, 600, and 1,100 nucleotides long, map between 3.21 and 4.24, 6.54 and 7.16, and 7.20 and 8.23 kbp from the end of the genome, and direct the cell-free translation of the 7.5, 19, and 42K polypeptides. Furthermore, these RNAs appear to be unspliced (33) and to have separate promoters (10). We found that the

TABLE 2. *Cap analysis of in vitro RNAs*

| Restriction fragment[a] (bp map units) | RNA[b] | % Total caps[c] | |
|---|---|---|---|
| | | G cap | A cap |
| 1. 3,390–4,350 | 7.5K | 78 | 22 |
| 2. 3,690–4,350 | 7.5K | 78 | 22 |
| 3. 3,735–4,350 | 7.5K | 81 | 19 |
| 4. 5,130–7,200 | 19K | 31 | 69 |
| 5. 5,280–7,200 | 19K | 24 | 76 |
| 6. 7,200–8,760 | 42K | 53 | 47 |
| 7. 8,760–9,000 | 21K | —[d] | 100 |

[a] The restriction fragments of the indicated map units were obtained by electrophoresis of: 1) *SalI* digest of pAG4 DNA, 2) *AluI* digest of pAG4 DNA, 3) *DdeI* digest of pAG4 DNA, 4) *HpaII* digest of λA7/1 DNA, 5) *HpaII* digest of pAGI DNA, 6) *HpaII* digest of λA7/1 DNA, and 7) *HpaII* digest of λA7/1 DNA. DNA fragments were transferred to diazotized paper by blotting.

[b] RNA synthesized in vitro by vaccinia virus cores was β-eliminated, enzymatically recapped using [α-$^{32}$P]GTP, hybridized to immobilized restriction fragments, RNase A treated, and eluted. The RNA is designated by the polypeptide it encodes. For no. 7, the polypeptide product was identified by Wittek and Cooper (personal communication).

[c] The percentages of A caps and G caps were determined as described in Table 1, footnote *a*.

[d] —, None.

three mRNA's made in vitro and in vivo have similar lengths and map positions and direct the synthesis of similar polypeptides in a reticulocyte cell-free extract. Thus, the virus core system appears to be well-suited for studying transcriptional mechanisms. In addition, the more detailed analysis used here provided finer mapping of the mRNA for the 7.5K polypeptide and also revealed that the 5′ end of a previously undescribed RNA maps beyond 8.7 kbp from the end of the genome; presumably its 3′ end is beyond the first *Eco*RI site and lies in the adjacent restriction fragment.

Analysis of the 5′ ends of total RNA synthesized in vitro by vaccinia virus indicated that they have m$^7$G(5′)pppA$^m$ or m$^7$G(5′)pppG$^m$ ends (19, 31). Further investigations indicated that all four ribonucleotides follow the A$^m$ and G$^m$ residues (15) and at least 40 RNase T1-capped oligonucleotides were resolved by two-dimensional electrophoresis (J. M. Keith and B. Moss, unpublished data). It is not yet certain whether capping occurs exclusively at the site of initiation of RNA synthesis, or also at a site of endonucleolytic cleavage. Incorporation of [β-$^{32}$P]GTP and [β-$^{32}$P]ATP into cap structures of total RNA has been demonstrated (19). Since only the α-phosphate of GTP is transferred by the RNA guanylyltransferase (17, 19), the radioactive β-phosphate of the cap must be derived from the initial ribonucleoside triphosphate forming the RNA. On the other hand, Paoletti (22) described conditions under which high-molecular-weight RNA is formed in vitro by vaccinia virus cores and is subsequently cleaved to mRNA size and suggested that internal sites might be capped. This possibility was supported by the finding of both an endoribonuclease activity (24) and a novel kinase that can add a second phosphate to the end of an RNA chain (26) in vaccinia virus cores. The latter enzyme transfers the γ-phosphate of ATP to RNA that ends in a single phosphate. At this stage of phosphorylation, the RNA could be capped by RNA guanylyltransferase (28). Thus, although some RNAs are capped at sites of initiation, it is possible that others are capped at sites of cleavage. It was of interest, therefore, to determine which mechanism is used to form the individual mRNA's encoded within the inverted terminal repetition. We found that all three mRNA's were labeled with [β-$^{32}$P]GTP, indicating that capping occurs at the sites of initiation of these RNAs. The transcriptional requirement for high ATP concentration precluded similar experiments with [β-$^{32}$P]ATP.

Analysis of the cap structures of both in vivo and in vitro RNAs revealed another interesting feature. Three appeared to have both m$^7$G(5′)pppG$^m$ and m$^7$G(5′)pppA$^m$ caps; the percentages were about 75%, 25%, and 50% m$^7$G(5′)pppG$^m$ for the mRNA's encoding 7.5, 19, and 42K polypeptides, respectively. However, only an m$^7$G(5′)pppA$^m$ cap was found in the additional RNA that is only partially encoded within the cloned DNA segment. The finding of two cap structures implies some degree of 5′-terminal heterogeneity. This heterogeneity may be similar to that found in the late mRNA's transcribed from polyoma (12) and simian virus 40 (8). However, the latter are transcribed by the cellular RNA polymerase, whereas the polypeptide subunits of the RNA polymerase from vaccinia virions suggest that it is a viral enzyme (2). Efforts are currently being made to sequence parts of the vaccinia virus genome and the ends of corresponding RNAs to determine the promoter recognition signals for the viral RNA polymerase.

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