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Intermolecular Duplexes Formed from Polyadenylylated Vaccinia Virus RNA

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Approximately 15% of the polyadenylic acid-containing cytoplasmic RNA labeled from 5 to 7 h after vaccinia virus infection formed intermolecular duplex structures characterized as double-stranded RNA by RNase resistance, density in Cs_2SO_4, base composition, chromatography on cellulose, and ability to inhibit reticulocyte cell-free protein synthesis. Both sucrose gradient sedimentation and electron microscopic analysis indicated that the double-stranded regions were several hundred to more than a thousand nucleotide base pairs long. The double-stranded RNA, after denaturation, hybridized to approximately 25% of the vaccinia virus genome, whereas total late RNA hybridized to 42%. The finding that the duplex RNA, after denaturation, hybridized to most *Hin*dIII restriction endonuclease fragments of vaccinia virus DNA indicated that symmetrical transcription is not confined to the terminal inverted repeat sequence or to one contiguous region of the genome. Although relatively little labeled, early, polyadenylic acid-containing RNA formed RNase-resistant hybrids upon self-annealing, the percentage increased upon addition of unlabeled late RNA, indicating that the latter contains "anti-early" sequences.

Vaccinia virus DNA is transcribed within the cytoplasm of infected cells (3) by virion-associated enzymes (19, 22). The mRNA is further modified to contain eucaryotic features including a 3'-terminal polyadenylic acid [poly(A)] sequence (21, 23, 27) and a 5'-terminal cap structure (5, 31). Hybridization of RNA to DNA immobilized on nitrocellulose filters suggested that early or prereplicative RNA has half the sequence complexity of late mRNA (24). In agreement with this result, approximately 26% of vaccinia virus DNA forms hybrids with excess early RNA, whereas 40 to 50% forms hybrids with excess late RNA (6, 20, 25). Other studies (9-11) revealed that virus-specific double-stranded RNA is present at both early and late times after vaccinia virus infection.

In the present communication, we show that approximately 15% of the polyadenylylated [poly(A)+] RNA present late after vaccinia virus infection is capable of annealing to form intermolecular duplexes. The latter double-stranded RNA has been characterized with regard to size, base composition, density, sequence complexity, and genome location.

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MATERIALS AND METHODS

Virus infections. HeLa S-3 cells were grown in suspension culture, sedimented, resuspended at a concentration of 5×10^6 cells per ml, and infected with 15 PFU of trypsin-treated unpurified vaccinia virus per cell as previously described (6). After a 30-min virus adsorption period, the cells were diluted 10-fold. In some experiments, cytosine arabinoside (40 μg/ml) was added 15 min before virus infection and maintained at this concentration to prevent viral DNA replication and late RNA synthesis (6).

Isolation of poly(A)+ RNA. At either 2 h (early) or 7 h (late) after addition of virus, cells were washed twice with cold isotonic saline and suspended in hypotonic buffer containing 10 mM Tris-hydrochloride (pH 7.6)-10 mM NaCl-1.5 mM $MgCl_2$ for 15 min at 0°C. After Dounce homogenization and centrifugation at 350 × *g* to remove nuclei, Sarkosyl was added to the cytoplasmic fraction to a final concentration of 2%. CsCl (1 g/ml) was added, and the solution was layered over a 1.5-ml cushion of 5.7 M CsCl-0.1 M EDTA (pH 7.6). After centrifugation for 15 h at 35,000 rpm in an SW41 rotor, the protein had formed a gel at the top of the tube and the DNA had banded above the cushion (6, 15). Both the protein and the DNA were carefully removed and the RNA pellet was dissolved in 0.1 M NaCl-10 mM Tris-hydrochloride (pH 7.6) and stored overnight in 75% ethanol at −20°C. (The separation of RNA from DNA was checked by labeling cells with [^{3}H]thymidine from 1 to 7 h after vaccinia virus infection and then subjecting the cytoplasmic fraction to the above procedure.) After centrifugation, the RNA

was dissolved in NETS buffer (0.12 M NaCl-2 mM EDTA-10 mM Tris-hydrochloride [pH 7.6]-0.1% sodium dodecyl sulfate) and applied to a polyuridylic acid [poly(U)]-Sepharose column (0.7 by 6 cm) equilibrated with NETS buffer. The column was washed with 50 ml of NETS buffer, and the poly(A)+ RNA was eluted with 90% formamide-10 mM Tris-hydrochloride (pH 7.6). The fractions containing poly(A)+ RNA in 90% formamide were incubated at 68°C for 2 min to disrupt any hybrid structures and then diluted with 5 volumes of NETS buffer and immediately applied to a second poly(U)-Sepharose column. After washing with NETS buffer, the poly(A)+ RNA was eluted with 90% formamide as before and precipitated with ethanol. The RNA was dissolved in 10 mM Tris-hydrochloride (pH 7.6), and the concentration was determined spectrophotometrically assuming that 45 μg of RNA per ml has an absorbancy at 260 nm of 1.

Isolation of RNA synthesized in vitro. RNA was synthesized in vitro by vacinia virus cores as previously described (6). For peparation of radioactively labeled RNA, 3 mM ATP-1 mM GTP-1 mM CTP-50 μM [^{3}H]UTP (600 mCi/mmol) was used. After 2 h at 37°C, virus cores were removed by sedimentation, and the RNA was further purified by centrifugation through CsCl and by poly(U)-Sepharose chromatography as described above.

Determination of RNase-resistant RNA. Before hybridization, [^{3}H]uridine-labeled poly(A)+ RNA samples were incubated in 10 mM Tris-hydrochloride (pH 7.6) for 2 min at 100°C. Hybridization at 68°C was in sealed capillary tubes containing 20 μg of yeast RNA in 0.1 ml of 1 M NaCl-10 mM Tris-hydrochloride (pH 7.6). At appropriate times, three 30-μl samples were taken from each capillary tube and mixed with 1 ml of 0.3 M NaCl-0.03 M sodium citrate. Duplicate samples were digested with a combination of RNase A (50 μg) and RNase T1 (2.5 μg) for 1 h at 37°C. After addition of 0.1 mg of bovine serum albumin and 10 ml of 5% trichloroacetic acid to each of the three samples, the precipitates were collected on glass fiber filters. The filters were dried and counted in a toluene-based scintillation fluid. Zero-time backgrounds of less than 1% were subtracted from values obtained at later times.

Isolation of double-stranded RNA. Two procedures were used to isolate double-stranded RNA after formation of RNA-RNA hybrids. Method A was used except where otherwise indicated. This procedure consisted of digesting [^{3}H]uridine-labeled RNA with RNase A (50 μg/ml) and RNase T1 (2.5 μg/ml), as described above, followed by phenol-chloroform (1:1) extraction and ethanol precipitation. ^{32}P-labeled RNA was further treated with 2 U of RNase T2 per ml in 0.3 M NaCl-10 mM ammonium acetate (pH 4.5) for 1 h at 37°C to digest poly(A). After filtration through Sephadex G-75, the RNA in the void volume was pooled and ethanol precipitated. Samples of the latter material were completely resistant to further RNase digestion.

Method B consisted of digesting the annealed RNA with lower concentrations of RNase (10 μg of RNase A per ml, 0.5 μg of RNase T1 per ml) at 37°C for 60 min followed by phenol-chloroform extraction, ethanol precipitation, and gel filtration through Sephadex G-75. The RNA in the void volume was ethanol precipitated and then redigested with RNase A and RNase T1 followed by chromatography on a column (4 by 0.7 cm) of CF11 cellulose at approximately 22°C as described by Franklin (12). The RNA sample was applied in 1 ml of 35% ethanol-0.12 M NaCl-2 mM EDTA-10 mM Tris-hydrochloride (pH 7.0), and the column was washed successively with 15 ml of the above, 10 ml of the above containing 15% ethanol, and then with 10 ml of buffer containing no ethanol. One-milliliter fractions were collected. Samples of the double-stranded RNA eluting at the last step were resistant to digestion with 50 μg of RNase A and 2.5 μg of RNase T1 per ml.

DNA-RNA hybridization. [^{3}H]thymidine-labeled vaccinia virus DNA, with a specific activity of 5.4 × 10^6 cpm/μg, was prepared as described (6). Hybridization of the DNA to unlabeled RNA was carried out at 68°C in 1 M NaCl, and the formation of hybrids was assayed by resistance to digestion with nuclease S1 (6).

Hybridization of labeled RNA to DNA restriction fragments transferred to nitrocellulose sheets by the method of Southern (28) was carried out as described previously (14).

Electron microscopy. Poly(A)+ RNA, at a concentration of 80 μg/ml, was incubated at 37°C for 2 h in 0.4 M NaCl-1 mM EDTA-40 mM HEPES (pH 6.7; *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid)-50% formamide and then mounted for electron microscopy either by the Kleinschmidt aqueous procedure or in formamide (32, 33). The 50-μl spreading solution contained 90% formamide-4.5 M urea-0.2 μg of RNA per ml-50 μg of cytochrome *c* per ml and was spread upon a distilled water hypophase at 4°C. Molecules were picked up on Parlodion-coated grids, stained with uranyl formate, and rotary shadowed with platinum-palladium. Grids were examined in a Siemens Elmiskop 1A electron microscope at 40-kV accelerating voltage. Electron micrographs were taken on Kodak electron image plates at ×6,000 to ×8,000. The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam no. 1000), and contour lengths were measured with a Numonics graphic calculator interfaced to a Wang 2200 computer. Double-stranded RNA was considered to be 2.1 × 10^6 daltons/μm (30). The absence of DNA in RNA samples for electron microscopy was checked by incubating preparations in 0.3 M KOH at 37°C for 18 h. After neutralization, the samples were spread and examined with the electron microscope.

RESULTS

Formation of RNase-resistant RNA. Previous studies (10) indicated that approximately 2.5% of pulse-labeled total cytoplasmic RNA obtained at late times after vaccinia virus infection of HeLa cells formed RNase-resistant hybrids. It was of interest to determine whether mutually complementary sequences are present in poly(A)+ RNA. Since 90% of early and late vaccinia virus mRNA contains poly(A) (23), se-

lection of RNA on a poly(U)-Sepharose column provides a significant enrichment in virus-specific sequences. Indeed, most of the poly(A)+ cytoplasmic RNA obtained late and up to half of that obtained early in infection may be virus specific (6).

Cells were labeled with [^{3}H]uridine from 5 to 7 h after infection. The cytoplasmic fraction was treated with Sarkosyl, and the RNA was centrifuged through CsCl to remove DNA. The poly(A)+ fraction of the RNA was then isolated by two rounds of poly(U)-Sepharose chromatography. Between the first and second chromatography steps, the RNA was heated at 68°C in 90% formamide to disrupt any hybrid structures. When self-annealed, with or without an additional DNase step, 12 to 18% of the poly(A)+ RNA was resistant to RNase digestion (Fig. 1A). The kinetics suggested that the major reaction was formation of intermolecular hybrids. Despite the use of considerably higher RNA concentrations, less than 2% of poly(A)+ RNA from similarly labeled uninfected cells or from cells labeled from 0 to 2 h after infection in the presence of cytosine arabinoside formed RNase-resistant hybrids (Fig. 1A). Similarly, only a low amount of poly(A)+ RNA synthesized in vitro by vaccinia virus cores formed hybrids (Fig. 1A).

Addition of unlabeled poly(A)+ late RNA "driver" to labeled late RNA accelerated the rate of formation of RNase-resistant hybrids but did not affect the final plateau value (Fig. 1B).

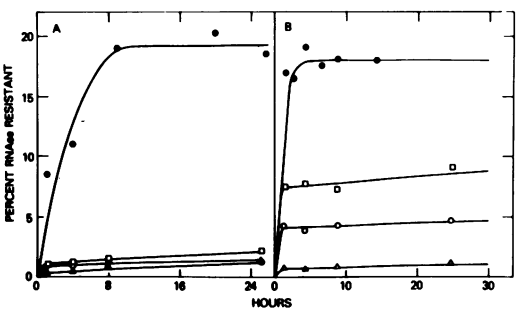

FIG. 1. *RNase resistance of RNA after annealing. (A) The following poly(A)+ RNA preparations were denatured and then annealed: (●) 3.1 μg of late RNA per ml labeled with [^{3}H]uridine from 5 to 7 h after infection; (□) 56 μg of early RNA per ml labeled with [^{3}H]uridine from 0 to 2 h after infection in the presence of cytosine arabinoside; (△) 70 μg of uninfected HeLa cell RNA per ml labeled for 2 h with [^{3}H]-uridine; and (○) 86 μg of in vitro synthesized vaccinia virus RNA per ml labeled with [^{3}H]UTP. (B) The labeled RNA preparations described above were annealed in the presence of 40 μg of unlabeled poly(A)+ RNA per ml obtained 7 h after vaccinia virus infection.*

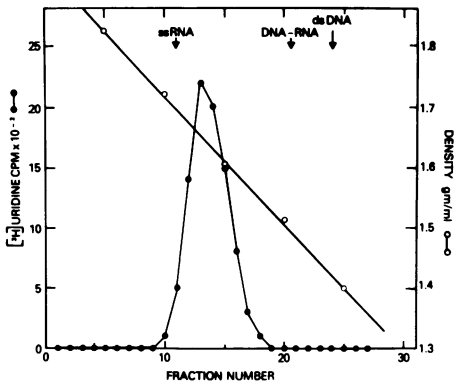

FIG. 2. *Density of double-stranded (ds) RNA. Poly(A)+ RNA was purified from vaccinia virus-infected HeLa cells labeled with [^{3}H]uridine from 5 to 7 h after infection. After digestion with RNases A and T1, the resistant material that was excluded from Sephadex G-75 was dissolved in Cs_2SO_4 with a density of 1.60 g/ml and centrifuged in an SW41 rotor at 35,000 rpm for 96 h. The following markers were applied to parallel gradients: [^{14}C]uridine-labeled total cytoplasmic HeLa cell RNA (single-stranded RNA [ssRNA]); [^{3}H]thymidine-labeled vaccinia virion DNA (dsDNA); and [^{3}H]thymidine-labeled vaccinia virus DNA-RNA hybrids isolated after digestion with nuclease S_1 (DNA-RNA).*

Addition of late poly(A)+ RNA driver, however, increased the percentage of hybrids formed with labeled early RNA and in vitro RNA to approximately 10 and 5%, respectively (Fig. 1B). As expected, late RNA driver had no effect on the formation of RNase-resistant hybrids with uninfected cell RNA (Fig. 1B). Further experiments indicated that addition of 65 μg of unlabeled early RNA per ml did not affect the level of RNase-resistant hybrids formed with labeled early, late, or in vitro RNA. These experiments suggested that mutually complementary RNA sequences are present in poly(A)+ RNA obtained at late times after infection and that some late RNA sequences are complementary to early RNA and to RNA synthesized in vitro by virus cores.

Characterization of double-stranded RNA. The RNase-resistant material, obtained after self-annealing of [^{3}H]uridine-labeled late poly(A)+ RNA, was excluded from Sephadex G-75 and further analyzed by isopycnic centrifugation in Cs_2SO_4. A density of 1.65 g/ml, which was appropriate for double-stranded RNA (29) and similar to the value previously obtained (10), was determined (Fig. 2). Significantly, no labeled material was detected at the density of DNA-RNA hybrids.

The RNase-resistant material was further

identified as double-stranded RNA by its chromatographic properties on cellulose (Materials and Methods [12]) and by its ability to inhibit rabbit reticulocyte protein synthesis in vitro (16). Addition to a reticulocyte lysate at zero time of only 1 ng of purified RNase-resistant RNA per ml, obtained from poly(A)+ late RNA, inhibited [^{35}S]methionine incorporation with biphasic kinetics as described by Hunter et al. (16). Maximum inhibition was obtained with approximately 30 ng of RNA per ml. Moreover, the inhibition could be specifically prevented by addition of either cyclic AMP (6 mM) or high concentrations (20 μg/ml) of double-stranded reovirus RNA (16). Hunter et al. (16) point out that only properly and extensively matched double-stranded RNA greater than 50 base pairs has these properties and that no forms of DNA, single-stranded RNA, or even RNA-DNA hybrids act as inhibitors in this way.

The base composition of the double-stranded RNA was determined to ensure that it is not poly(A):poly(U) or another unusual sequence. For this analysis, cells were labeled with $^{32}P_i$ from 5 to 7 h after infection. The poly(A)+ RNA was isolated as previously described, self-annealed, and then digested with RNases A and T1, followed by RNase T2 to digest single-stranded poly(A). After gel filtration, the excluded material was hydrolyzed with KOH and subjected to paper electrophoresis at pH 3.5. The results (31.9% Ap, 32.2% Up, 18.4% Gp, 17.5% Cp) indicated similar amounts of Ap and Up and of Gp and Cp, as expected for a base-paired double-stranded structure.

Sequence complexity of double-stranded RNA. Double-stranded RNA obtained by RNase treatment and gel filtration of self-annealed late poly(A)+ RNA was heated to dissociate the strands and then hybridized to denatured [^{3}H]thymidine-labeled vaccinia virus DNA. The fraction of the DNA that formed hybrids was determined by digestion with nuclease S1. In the experiment shown in Fig. 3, approximately 26% of the DNA hybridized in 3 h and no further hybridization occurred by 12 h. With three separate preparations of double-stranded RNA, 24.5 ± 2.8% of the DNA formed DNA-RNA hybrids. In parallel experiments, we confirmed our previous report (6) that more than 40% of the DNA formed hybrids with total poly(A)+ late RNA.

To determine whether the 25% of the genome that is symmetrically transcribed is localized within a specific region, the DNA was cleaved with *Hin*dIII restriction endonuclease (35). The 15 fragments obtained from strain WR vaccinia virus DNA were separated by electrophoresis in a composite 0.6 to 1.5% agarose gel and transferred to a nitrocellulose sheet by the procedure of Southern (28) as described previously (14). All fragments were able to hybridize to total cytoplasmic poly(A)+ late RNA as previously reported (8), and most fragments were labeled with denatured double-stranded RNA (Fig. 4). Although relatively less double-stranded RNA than total RNA appeared to hybridize to fragments F, I, K, and O, more quantitative studies would be required to further evaluate this. The major point we wish to make is that the double-stranded RNA does not appear to be transcribed exclusively from a limited region of the DNA genome, such as the inverted terminal repetition (13, 34) which is present in fragments B and C (13).

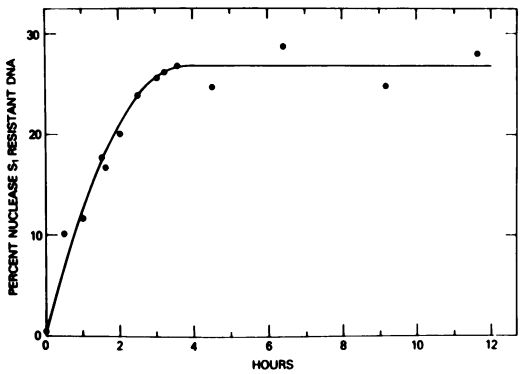

FIG. 3. *Sequence complexity of double-stranded RNA. Excess double-stranded RNA and [^{3}H]thymidine-labeled vaccinia virus DNA (5.4 × 10^6 cpm/μg) were denatured at 100°C for 5 min, and the formation of hybrids was analyzed by digestion with nuclease S_1. Corrections for low levels of DNA-DNA annealing in parallel incubations have been made.*

Sedimentation of RNA-RNA hybrids. After self-annealing, [^{3}H]uridine-labeled late RNA sedimented as a broad, heterogeneous peak with a maximum at 14S (Fig. 5). The fraction of the RNA that was RNase resistant increased with increasing sedimentation coefficient to a maximum of 25 to 27% (Fig. 5), whereas total RNA was about 15% RNase resistant.

The sedimentation behavior of the double-stranded RNA was more unform after RNase digestion, suggesting the removal of variable length, single-stranded tails. Double-stranded RNA isolated by method A sedimented at approximately 8 to 10S (data not shown), consistent with previous results (10). Using isolation method B, which involved lower concentrations of RNase but included cellulose chromatography to remove any RNA with undigested single-stranded tails, the duplex RNA sedimented at

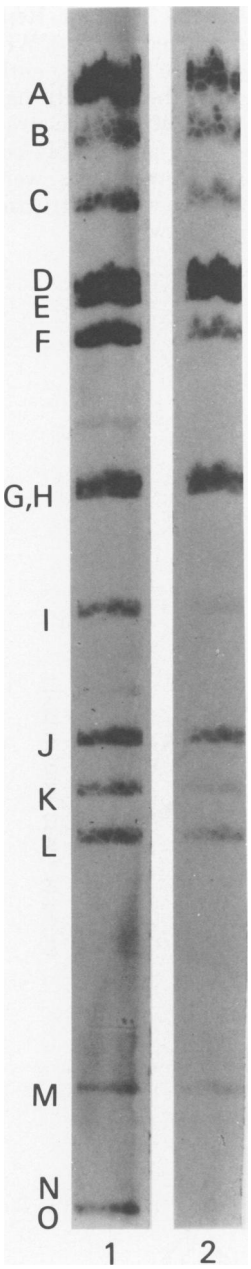

FIG. 4. *Hybridization of double-stranded RNA to vaccinia virus DNA restriction fragments. Double-stranded RNA was prepared by method B, which included cellulose chromatography, from cells labeled with [^{3}H]uridine from 5 to 7 h after infection. The RNA was denatured and annealed to electrophoretically separated HindIII restriction fragments of vaccinia virion DNA that had been transferred to nitrocellulose sheets. Fluorograms are shown. (1) Total cytoplasmic poly(A)+ RNA; (2) double-stranded RNA.*

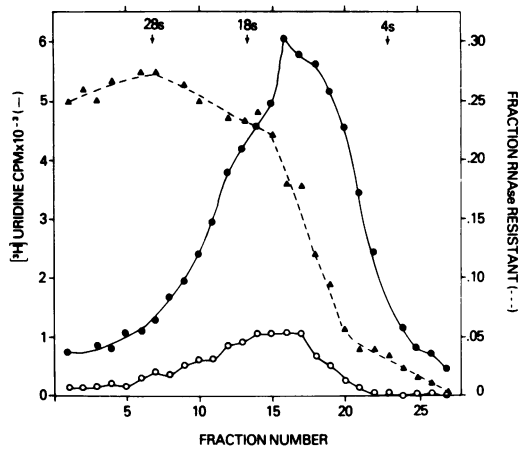

FIG. 5. *Sucrose gradient sedimentation of annealed RNA. Poly(A)+ RNA was purified from the cytoplasm of cells labeled with [^{3}H]uridine from 5 to 7 h after infection and was annealed in 1 M NaCl for 4 h at 68°C. After sedimentation in a 5 to 20% sucrose gradient, duplicate portions of each fraction were precipitated with trichloroacetic acid and counted directly (●) or after digestion with RNases A and T1 (○), and the resistant fraction (▲) was calculated. The positions of rRNA and tRNA markers were determined in a parallel gradient.*

13S (Fig. 6A). After denaturation, the latter RNA sedimented at approximately 10S (Fig. 6B). In contrast, double-stranded RNA prepared by method A sedimented at less than 4S after denaturation (not shown). Evidently, the higher concentrations of RNase used in method A either cleaved at small single-strand gaps or within the duplex structure itself.

Visualization of RNA-RNA hybrids. For visualization of duplex RNA structures by electron microscopy, annealing was performed at 37°C in 50% formamide instead of at 68°C to minimize RNA degradation. Nevertheless, the formamide hybridization conditions did not significantly alter the fraction of the cytoplasmic late poly(A)+ RNA that became RNase resistant. When the self-annealed RNA was mounted for electron microscopy using the aqueous spreading technique, approximately 15% of the molecules appeared as double or partially double-stranded rods and the rest as collapsed bushlike structures (Fig. 7B). The mean length of 61 measured duplex rods was 0.27 μm, with a range of 0.06 to 0.60 μm (Fig. 8). These lengths correspond to approximately 200 to 2,000 base pairs, with a mean of 860. After denaturation and before annealing, the vast majority of structures appeared as collapsed bushlike structures, although some short rods, possibly representing intramolecular duplexes, were seen (Fig. 7A).

RNA isolated early after infection also appeared primarily as collapsed structures even after annealing (not shown).

Additional structural information was obtained by spreading the self-annealed late RNA in 90% formamide–4.5 M urea (32, 33). Under these stringent conditions, single-stranded RNA is not collapsed but still can be distinguished from double-stranded RNA by)ts thinner appearance and less rigid contour. Representative structures are displayed in Fig. 9. Whereas some structures appeared to be almost entirely double stranded (Fig. 9A), the majority had one (Fig. 9B) or two (Fig. 9C) single-stranded tails. Branched structures, presumably composed of more than two RNA molecules, were also seen (Fig. 9D). Some large duplex structures had six or more tails (not shown).

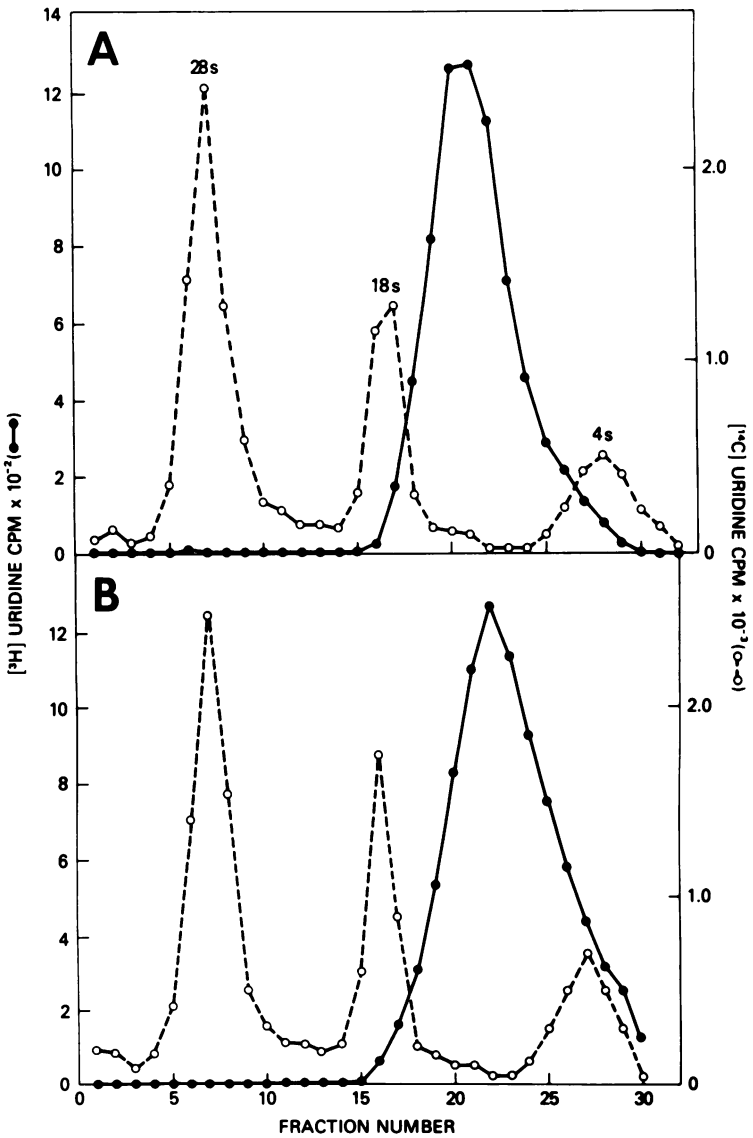

FIG. 6. *Sucrose gradient sedimentation of double-stranded RNA. Double-stranded RNA was obtained by RNase digestion of annealed [³H]uridine-labeled late poly(A)+ RNA and purified by gel filtration and cellulose chromatography. (A) Double-stranded RNA was sedimented in a 5 to 20% sucrose gradient with ¹⁴C-labeled rRNA and tRNA marker. (B) Double-stranded RNA was heated at 100°C for 2 min just before centrifugation.*

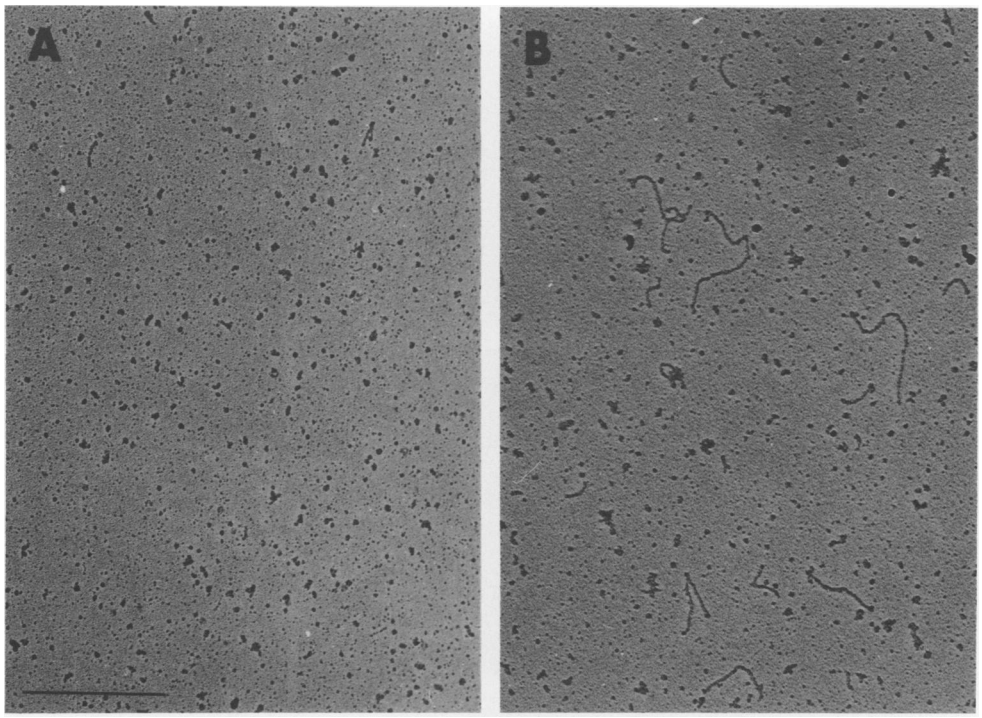

FIG. 7. *Electron micrographs of poly(A)+ RNA spread by the Kleinschmidt aqueous technique to reveal duplex structures. Poly(A)+ RNA, purified from the cytoplasm of HeLa cells at 7 h after infection, was examined after (A) heat denaturation and (B) self-annealing for 2 h. ×18,000; bar = 1 μm.*

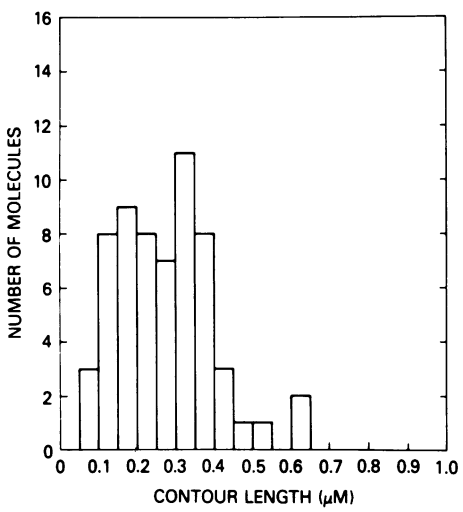

FIG. 8. *Contour length measurements of double-stranded RNA molecules spread by the Kleinschmidt aqueous technique.*

DISCUSSION

Symmetrical transcription has been reported for a variety of animal viruses, including vaccinia virus (9–11), polyoma virus (2), simian virus 40 (1), adenovirus (26) and herpesvirus (17), as well as for some bacteriophages such as λ (7) and T4 (18). Our results with vaccinia virus confirm and extend previous studies (9–11). Specifically, we find that cytoplasmic poly(A)+ RNA obtained late after vaccinia virus infection can anneal to form duplex structures. After CsCl centrifugation to remove DNA, the RNA isolation procedure involved heating at 68°C in 90% formamide between the first and second rounds of poly(U)-Sepharose chromatography. Therefore, it seems unlikely that only one of the complementary strands contains poly(A) and that the other was carried along with it. The double-stranded RNA, formed after annealing, was identified by its RNase resistance, density in Cs_2SO_4, base composition, chromatography on cellulose columns, and ability at low concentration to inhibit reticulocyte cell-free protein synthesis. Since greater than 90% of the total vaccinia virus-specific RNA in infected cells has been reported to contain poly(A) (23), we have focused our attention on this fraction. The possibility that additional double-stranded RNA could be formed from RNA that does not contain poly(A) has not been examined.

Approximately 15% of poly(A)+ RNA labeled

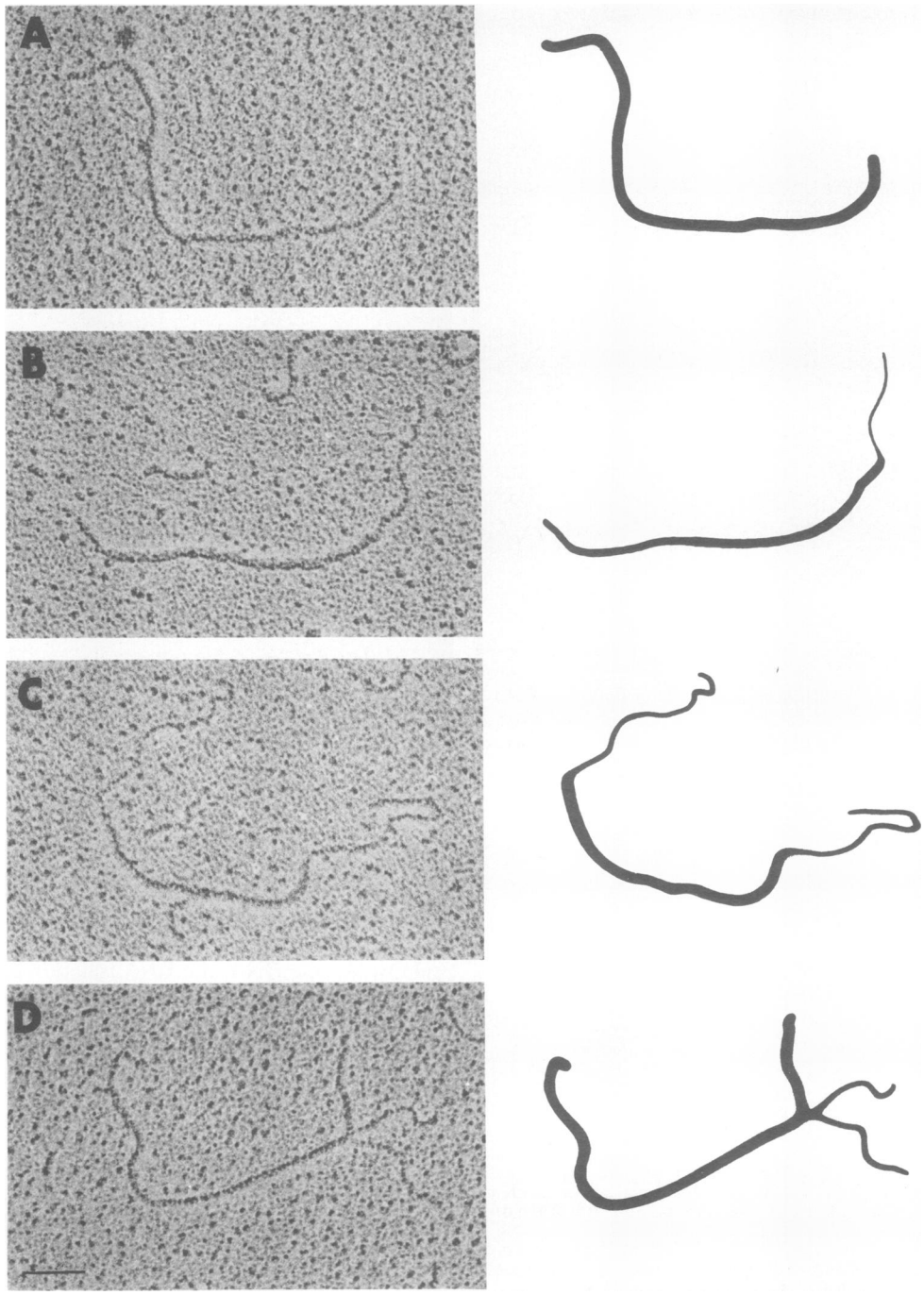

FIG. 9. *Electron micrographs of annealed poly(A)+ RNA spread in 90% formamide–4.5 M urea to resolve double- and single-stranded molecules. RNA similar to that shown in Fig. 7 was self-annealed for 2 h and mounted in 90% formamide–4.5 M urea as described in the text. Electron micrographs and drawings of representative molecules exhibiting duplex structures are shown in panels A through D. ×80,000; bar = 0.1 μm.*

from 5 to 7 h after vaccinia virus infection formed duplex structures. Considerably less double-stranded RNA was formed when poly(A)+ early RNA or poly(A)+ RNA synthesized in vitro by vaccinia virus cores was self-annealed. Although Colby and co-workers (10) also found

maximal amounts of double-stranded RNA to be present at late times, the percentage of RNase-resistant RNA was lower in their experiments, presumably because total cytoplasmic RNA was used rather than poly(A)+ RNA, which is considerably enriched in virus-specific sequences.

The sedimentation value of the RNase-resistant duplex RNA suggested a molecular weight of at least 10^6 (4) equivalent to 1,500 nucleotide base pairs, whereas a mean lengh of 860 nucleotide base pairs was estimated from electron microscopic contour length measurements. The lower value obtained by electron microscopy may be due in part to our selection of only unbranched molecules for measurement. Although a minority of structures appeared as almost complete duplexes, most had single-stranded tails and some appeared as complex branched structures composed of multiple RNA molecules. The more complex structures could form from RNA molecules of overlapping length produced either in vivo or by degradation during sample preparation. The possibility of such structures resulting from spliced RNA molecules, however, has not been excluded.

Whereas excess late poly(A)+ RNA hybridized to 42 to 50% of the vaccinia virus genome (6, 20, 25), denatured double-stranded RNA obtained from the late poly(A)+ RNA only hybridized to 25% of the DNA. The sequence complexity of both the double-stranded RNA and the total late poly(A)+ RNA could be an underestimate, however, if some of the RNA sequences are present in low abundance. We had considered the possiblity that self-annealing RNA could be derived by transcription of the inverted terminal repetition. However, the repetition comprises only 10% of total vaccinia virus DNA (13, 34). Moreover, hybridization of labeled denatured double-stranded RNA sequences to *Hin*dIII endonuclease restriction fragments of vaccinia virus DNA indicated that the transcribed regions are not located exclusively within the inverted terminal repeat sequence or another limited area of the genome.

There is little information regarding the mechanisms of synthesis of the mutually complementary RNA species (10). Whether the synthesis of mutually complementary RNA strands is biologically important or results from initiation errors, improper termination, or incomplete processing of transcripts is unknown. A definitive answer to the question of whether both complementary strands are translatable will require the use of separated strands of vaccinia virus DNA restriction fragments to isolate the complementary RNA species. Preliminary experiments, however, suggest that some mutually complementary sequences are associated with polyribosomes (Boone, unpublished data).

Colby and co-workers suggested that some of the complementary RNA exists as duplex structures prior to extraction (10) and that the double-stranded RNA can induce the production of interferon (9). Our finding, that late RNA contains "anti-early" sequences, raises the possibility that formation of double-stranded RNA by annealing of late RNA with residual or continuously synthesized early RNA could play a role in shutoff of early protein synthesis.

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