*ON THE BIOSYNTHESIS AND STRUCTURE OF DOUBLE-STRANDED RNA IN VACCINIA VIRUS-INFECTED CELLS**

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Abstract.—The virus-specific, RNase-resistant RNA appearing in vaccinia virus-infected cells was directly shown to be an RNA duplex. After its melting and subsequent banding on Cs_2SO_4 or incubation with DNase, the RNA could be reannealed and then hybridized with vaccinia virus DNA.

The double-stranded virus-specific RNA appears to exist in the cell in the form of heterogeneous partially double-stranded RNA's sedimenting between 9 and 22*S*. The kinetics of the appearance of the double-stranded RNA in the cell show a dependence on the multiplicity of infection and suggest that the double-stranded RNA is a "late" product of viral intracellular biosynthesis. The synthesis of the double-stranded RNA is inhibited by actinomycin D.

It has recently been shown that vaccinia virus, which is a large animal virus containing DNA as its genetic material,[1] induces a virus-specific RNase-resistant RNA in infected chick cells.[2] The purified RNase-resistant RNA was found to have many properties of double-stranded RNA, to hybridize with vaccinia virus DNA, and to induce interferon.[2] In the present report, experiments are described which further support the notion that the virus-specific RNase-resistant RNA is a double-stranded RNA rather than a DNA-RNA hybrid. In addition, the kinetics of the biosynthesis of the double-stranded viral RNA were investigated.

Materials and Methods.—Standard buffer contains 0.1 *M* NaCl, 0.01 *M* Tris HCl pH 7.4, and 0.001 *M* EDTA. 5-^{3}H-uridine with a specific activity of 20 C/mmole was purchased from New England Nuclear.

Growth of virus: All studies on the replication of vaccinia virus (obtained from Dr. B. Woodson, University of California Medical Center, San Francisco) were done on one-day-old confluent chick embryo cell cultures in 10 cm plastic Petri dishes as described previously.[2]

Preparation of cytoplasmic RNA: All operations were carried out at 0–4°C. The procedure for cell fractionation has been described.[3] The RNA of a cytoplasmic extract was isolated by the phenol method[2] after the addition of EDTA to a final concentration of 0.025 *M*, sodium dodecylsulfate, and mercaptoethanol to 1%. After alcohol precipitation, the RNA was freed of slowly sedimenting ($\leqq$4-6S) RNA by chromatography on a 6 per cent agarose column which was described previously.[2]

Results.—Melting and subsequent self-annealing of the RNase-resistant RNA after elimination of DNA: In order to determine directly whether the RNase-resistant RNA of vaccinia-infected cells consisted of complementary RNA's rather than RNA-DNA hybrids, the following three experiments were carried out. Vaccinia virus-specific RNase-resistant RNA was labeled with ^{3}H-uridine and isolated from the total nucleic acids of infected cells by the method described

previously.[2] The RNase-resistant ^{3}H-RNA was then melted and annealed after the following operations (Table 1).

(1) The melted ^{3}H-RNA was mixed with 20 μg of tobacco mosaic virus RNA and annealed.

(2) The melted ^{3}H-RNA was separated from putative complementary vaccinia DNA by equilibrium sedimentation in Cs_2SO_4;[2] 20 μg of tobacco mosaic virus RNA were added, and the mixture was precipitated with ethanol. The RNA was then treated as described in (1) above.

(3) ^{3}H-vaccinia RNA (15,000 cpm) was combined with chick cell ^{14}C-DNA (24,000 cpm) in 250 μl 0.01 *M* NaCl, 0.01 *M* Tris (pH 7.4), and the mixture was melted at 100°C for 3 minutes. Fifty μg of RNase-free DNase (Worthington) and $MgCl_2$ to a final concentration of 2 mM were added, and the

TABLE 1. *Selfannealing of melted, double-stranded vaccinia virus RNA.*

	Melted vaccinia RNA (cpm)	RNase-resistant (cpm)	^{3}H-RNA after selfannealing (%)
1	2000	316	16
	2000	305	15
After Cs_2SO_4			
2	580	71	12.3
	580	58	10
After DNase			
3	740	90	12.2
	740	93	12.6
	1400	230	16.5
	1400	210	15

Experiment 1. Purified double-stranded ^{3}H-vaccinia RNA was isolated as described previously.[2] The RNA was melted in presence of 20 μg tobacco mosaic virus RNA by being boiled for 3 min in 0.01 *M* NaCl, 0.01 *M* Tris pH 7.4, and then lyophilized. It was then redissolved in 50 μl 0.25 *M* NaCl, 0.02 *M* Tris pH 7.4, and 1 mM EDTA, incubated in a sealed ampule for 5 min at 100°C, and allowed to cool slowly to 36°C over a period of 6 hr. The RNA was then incubated with p-RNase (20 μg/ml) and T_1-RNase (0.5 μg/ml) for 30 min at 37°C in 0.2 *M* NaCl, 0.02 *M* Tris pH 7.4, and 1 mM EDTA, and the TCA-precipitable radioactivity was determined as described for Fig. 1.

Experiment 2. Melted double-stranded ^{3}H-vaccinia virus RNA was banded to equilibrium in a Cs_2SO_4 density gradient as described previously.[2] The RNA was then selfannealed as in (1) above.

Experiment 3. Double-stranded ^{3}H-vaccinia RNA was incubated with DNase (see text) and then selfannealed as in (1).

solution was incubated for 2 hours at room temperature. After the addition of 1 ml standard buffer and EDTA to 5 mM, the solution was extracted once with 2 volumes of phenol and precipitated with ethanol. Ninety per cent of the ^{3}H-RNA (1620 cpm) and 3 per cent of the ^{14}C-DNA (890 cpm) were recovered, indicating that 97 per cent of the included ^{14}C-DNA was converted to TCA-soluble material. The RNA was then annealed (Table 1).

It can be seen in Table 1 that about 12–16 per cent of the melted input RNA could be reannealed under the conditions of the experiment and that the same percentage of melted ^{3}H-vaccinia RNA reannealed with or without prior elimination of DNA. It would appear that the original RNase-resistant RNA was double-stranded RNA free of DNA.

As a control, double-stranded influenza-virus RNA[4] was melted and annealed under the same conditions (Table 1); it was then found that 60 per cent of the

RNA could be reannealed. The difference between the annealing efficiency of double-stranded vaccinia RNA (12–16%) and influenza-virus RNA may be only reflective of the lower concentration, i.e., higher specific radioactivity of vaccinia RNA, but other explanations like greater heterogeneity of vaccinia RNA are possible.

Since only about 16 per cent of the RNase-resistant vaccinia RNA could be reannealed after melting (Table 1), and since not more than 33 per cent of the RNA hybridized with vaccinia DNA,[2] it is conceivable that the 16 per cent of the RNase-resistant RNA which could be self-annealed and the 33 per cent which could be hybridized to viral RNA were different species of intracellular RNA.

In order to determine whether the RNase-resistant RNA which can be reannealed after melting was virus-specific, the following experiment was carried out. Double-stranded ^{3}H-vaccinia virus RNA was melted and reannealed. The reannealed RNA was freed of single-stranded RNA by RNase digestion (Table 2). Subsequently, the RNase-resistant fraction was melted and hybridized with vaccinia virus DNA.[2] As is shown in Table 2, about 30

TABLE 2. *Hybridization of melted and selfannealed double-stranded vaccinia virus RNA to vaccinia virus DNA.*

cpm of melted RNA applied	cpm bound to 2 μg vaccinia DNA	cpm bound to empty filter
500	152	17
500	177	23

Double-stranded ^{3}H-vaccinia virus RNA (10,000 cpm) was melted and reannealed as described in the footnotes to Table 1. It was then incubated with RNase (30 min, 37°C 20 μg p-RNase/ml, 0.25 μg/ml T_1 RNase, 0.2 *M* NaCl, 1 mM $MgCl_2$, 0.01 *M* Tris pH 7.4), purified by three phenol extractions, and precipitated with alcohol after the addition of carrier 40 μg TMV RNA. The RNase-resistant fraction was then melted and hybridized to vaccinia-virus DNA by the method of Gillespie and Spiegelman[11] as described previously.[2]

per cent of the input RNA could be hybridized to vaccinia DNA. This experiment indicates that the double-stranded RNA is complementary to vaccinia-virus DNA.

Intracellular form of the RNase-resistant RNA: The RNase-resistant RNA of vaccinia-virus infected cells has been isolated as a rather homogeneous 11*S* component after RNase and DNase digestion followed by agarose chromatography of the total cellular nucleic acid.[2] Since vaccinia virus replicates in the cytoplasm of the cell,[1] and since almost no fast-sedimenting (>4–6*S*) cellular RNA's are transported from the nucleus to the cytoplasm in chick cells until about 20–30 minutes after incorporation of ^{3}H-uridine,[5, 6] all fast-sedimenting (>4–6*S*) pulse-labeled cytoplasmic RNA is thought to be virus-specific.[1, 7] In order to obtain a sedimentation distribution of the RNase-resistant RNA prior to digestion with RNase, the total cytoplasmic RNA of vaccinia-virus infected cells was pulse-labeled with ^{3}H-uridine for 15 minutes. The RNA was then isolated and was freed of 4–6*S* cellular RNA by chromatography on a 6 per cent agarose column (*Materials and Methods*) prior to sedimentation analysis.

It can be seen in Figure 1 that the total ^{3}H-RNA sedimented as a broad zone with a peak at about 12–14*S*. Similar sedimentation patterns for vaccinia messenger-RNA were previously described.[1] The RNase-resistant RNA had a

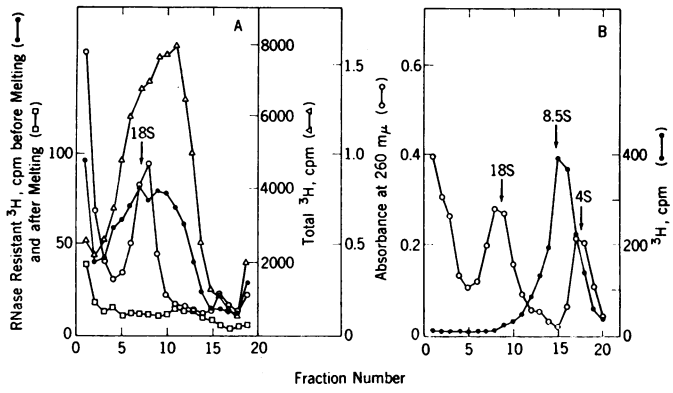


FIG. 1.—Sucrose gradient sedimentation of the cytoplasmic RNA of vaccinia-virus infected cells before (*A*) and after (*B*) digestion with RNase. Cells infected at a multiplicity of 50 were pulse-labeled at 4 hr post-infection with 100 μc ³H-uridine per culture for 15 min.

(*A*) Cytoplasmic RNA (see *Materials and Methods*) was dissolved in 0.2 ml standard buffer and centrifuged in a glycerol gradient (15–30% v/v) containing standard buffer for 200 min in a Spinco SW 65 rotor at 65,000 rpm and at 7°C. Four drop fractions were collected. Optical absorbance at 260 mμ (○) was determined after dilution of each fraction with 0.2 ml H_2O. Total ³H-radioactivity (△) was determined from a 50 μl aliquot by precipitation with an equal volume of 10% trichloroacetic acid (TCA) in the presence of 200 μg yeast RNA at 0°C. The precipitate was washed on Millipore filters. After 5 ml toluene-based scintillation fluid had been added to the dried filters, radioactivity was measured in TRICARB scintillation counter. Another 100 μl aliquot of each fraction was incubated in 0.2 *M* NaCl, 0.01 *M* Tris pH 7.4, and 2 mM $MgCl_2$ with RNase (20 μg/ml) and DNase (20 μg/ml) for 45 min at 37°C prior to determination of TCA-precipitable radioactivity (●) as described above. Another 100 μl aliquot was diluted tenfold with H_2O and heated for 5 min in boiling water and subsequently chilled in melting ice. It was then incubated with RNase and DNase under the above described conditions prior to determination of TCA-precipitable radioactivity (□).

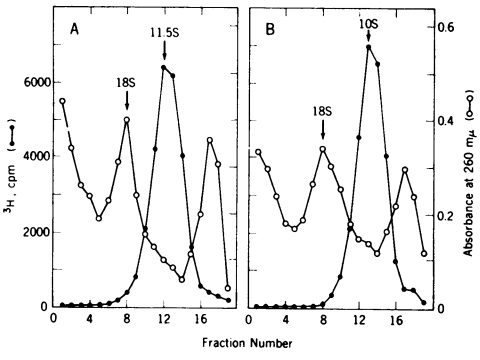
(*B*) A fraction of the cytoplasmic RNA used in (*A*) was incubated with RNase (20 μg/ml) and DNase (20 μg/ml) in 0.2 *M* NaCl, 0.01 *M* Tris pH 7.4, and 2 mM $MgCl_2$ for 30 min at room temperature. The RNase-resistant ³H-RNA (●) was then freed of degraded RNA by chromatography on an agarose column[2] and analyzed by sucrose gradient sedimentation as described in (*A*).

sedimentation distribution which differed from that of the total RNA. It had a broad maximum between 20 and 16*S*. The fraction of RNase-resistant RNA per total RNA in the gradient increased with increasing sedimentation coefficient of the RNA. About 1 per cent of the total cytoplasmic RNA of virus-infected cells was resistant to RNase and DNase. After melting for 5 minutes at 100°C in 0.01 *M* NaCl, 90 per cent of the RNase-resistant RNA was rendered sensitive to RNase and DNase (Fig. 1).

If an aliquot of the cytoplasmic RNA used in the experiment illustrated in Figure 1*A* was incubated for 30 minutes at room temperature with RNase and DNase as described previously[2] and then analyzed by sucrose gradient sedimentation, a single 8.5*S* component was obtained (Fig. 1*B*). This result is compatible with the previous sedimentation pattern of the RNase-resistant RNA of the unfractionated virus-infected cell where a single 11*S* component of RNase-resistant RNA was obtained. The lower sedimentation coefficient of 8.5*S* of

the RNase-resistant RNA obtained from the cytoplasmic extract may be the result of the conditions of the nuclease degradation. If the RNA is isolated from the whole cell, a large excess of cellular DNA and RNA is present during the nuclease digestion step of the isolation procedure.[2] This increases the nucleic acid/nuclease ratio and presumably prevents maximal degradation. This view is supported by the following experiment. RNase-resistant RNA of vaccinia-infected cells isolated as described previously[2] was divided into two aliquots. One aliquot was sedimented directly in the presence of cellular RNA (Fig. 2*A*). The other aliquot was sedimented after an additional incubation for 30 minutes with RNase and DNase in absence of DNA and in the presence of only small amounts of carrier TMV RNA. This treatment reduced the sedimentation coefficient of the RNase-resistant vaccinia RNA from about 11.5*S* to 10*S* (Fig. 2*B*).

From the experiments illustrated in Figures 1 and 2 it can be concluded that the double-stranded RNA in vaccinia-virus infected cells exists in the cell in relatively heterogeneous components with sedimentation coefficients ranging from about 9–22*S*. After extensive digestion with RNase, a rather homogeneous RNase-resistant core was obtained.



← Fig. 2

Fig. 3 →

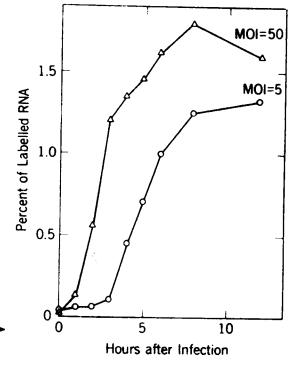


FIG. 2.—Dependence of the sedimentation coefficient of the double-stranded ^{3}H-RNA of vaccinia-virus infected cells upon condition of incubation with RNase. (*A*) Sedimentation pattern of a mixture of cell RNA (○) and of double-stranded vaccinia-virus ^{3}H-RNA (●) obtained by nuclease digestion of the total cellular nucleic acid and subsequent purification by chromatography on a 6% agarose column.[2] The ^{3}H-RNA was precipitated with ethanol in the presence of 100 μg cell RNA and redissolved in 0.2 ml standard buffer prior to sedimentation as described in Fig. 1*A*. (*B*) Sedimentation pattern of another aliquot of the same double-stranded ^{3}H-RNA (●) as used in (*A*) after an additional incubation in the presence of 40 μg of tobacco mosaic virus carrier-RNA with RNase and DNase under the conditions described previously.[2] After the nuclease digestion, EDTA was added to the reaction mixture to a final concentration of 0.01 *M* and SDS and mercaptoethanol to 1%, and the solution was extracted 3 times with phenol. The RNA was then precipitated with ethanol in the presence of 100 μg cell RNA, and was analyzed as described in (*A*).

FIG. 3.—Effects of the multiplicity of infection (MOI) on the kinetics of synthesis of vaccinia virus-specific double-stranded ^{3}H-RNA. Chick embryo cells (approximately 1.2×10^7 per culture) were pulse-labeled for 1 hr with 100 μc ^{3}H-uridine at various times after infection with vaccinia virus. The total nucleic acids were prepared by the phenol-SDS method and double-stranded RNA was prepared by nuclease digestion followed by chromatography on agarose as previously described.[2] The percentage of double-stranded ^{3}H-RNA of the total ^{3}H-RNA was determined at various times after infection at a multiplicity of infection of 5 (○) and of 50 (△).

Kinetics of appearance: The kinetics of the synthesis of the intracellular double-stranded RNA in the growth cycle of vaccinia virus were studied by pulse-labeling infected cells at various times after infection and isolation of the double-stranded RNA.[2] As seen in Figure 3, the time of the appearance of the double-stranded RNA in the growth cycle of the virus is dependent on the multiplicity of infection. At a multiplicity of infection of 50, the half maximal concentration was reached at about 2.5 hours, and at a multiplicity of 5, the half maximal concentration of intracellular RNase-resistant RNA was obtained at 5 hours post-infection. Dependence of intracellular events on the multiplicity of infection is typical for vaccinia virus replication.[1] The time course of the appearance of the double-stranded RNA in the cell is compatible with a "late" event of the intracellular viral growth cycle.[1]

Effects of actinomycin D on the synthesis of double-stranded vaccinia virus RNA: The synthesis of the double-stranded vaccinia-virus could proceed by two basically different mechanisms. It could be a DNA-dependent or an RNA-dependent mechanism of replication.

If the double-stranded vaccinia-virus RNA is directly transcribed from viral DNA, its replication would be inhibited by actinomycin D. This was tested in the following experiment. The cytoplasmic RNA of a vaccinia-virus infected cell was pulse-labeled with ^{3}H-uridine for 15 minutes at about 4 hours after infection (see Fig. 1). Two parallel experiments were done. In one, actinomycin D was added 30 minutes prior to the addition of the ^{3}H-uridine; the other was carried out without inhibitor. The purified cytoplasmic RNA (see *Materials and Methods*) of each of the two experiments was then incubated with RNase and DNase (Fig. 4) and analyzed by chromatography on an agarose column; there was no optically detectable nucleic acid in the exclusion volume (fractions 5 and 6) of the column, indicating that the nucleases had effectively degraded the cytoplasmic RNA's and DNA's. However, the profile of trichloroacetic acid-precipitable radioactivity shows a peak of ^{3}H-uridine labeled material in the exclusion volume (fractions 5 and 6) for the digest of the ^{3}H-RNA of both untreated (Fig. 1*B*) and actinomycin D-treated cells (Fig. 1*A*). As is shown in Table 3, actinomycin D inhibited the total RNA synthesis by about 98.8 per cent. The synthesis of RNase-resistant RNA was inhibited about 95 per cent by actinomycin D relative to total RNA synthesis in the absence of actinomycin D. The percentage of RNase-resistant RNA in untreated cultures was 0.8 per cent, which is compatible with similar values obtained previously,[2] but the percentage of RNase-reistant RNA of actinomycin D-treated cultures was about 2.8 per cent of the total. This experiment indicates that the synthesis of RNase-resistant RNA in vaccinia virus-infected cells is less inhibited by actinomycin D than is the synthesis of single-stranded RNA (about 95% and 98.9%, respectively). Very similar numbers were obtained when infected cells were labeled at about 4 hours postinfection for 1 hour with ^{3}H-uridine in the presence or absence of actinomycin D. The inhibition of total RNA synthesis by actinomycin D was 98 per cent, whereas the inhibition of RNase-resistant RNA was 96 per cent.

Discussion.—The result that the virus-specific RNase-resistant RNA of vaccinia-virus infected cells can be melted and reannealed after the elimination of

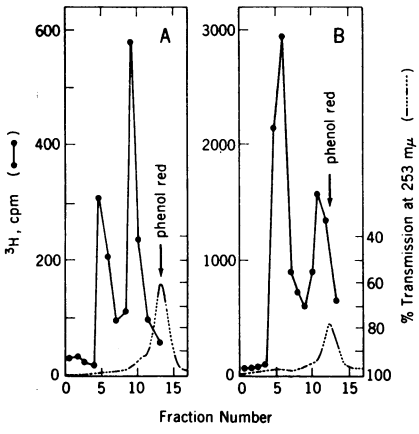


FIG. 4.—Agarose chromatography of the cytoplasmic ^{3}H-RNA (●) of vaccinia-virus infected cultures after digestion with RNase. The cytoplasmic RNA was labeled for 15 min with ^{3}H-uridine in presence (A) and absence (B) of actinomycin D.

(A) Actinomycin D (50 μg/5 ml) was added to the medium of vaccinia-virus infected cells (*Materials and Methods*) 4 hr post-infection. Thirty minutes later the culture was pulse-labeled with 100 μc ^{3}H-uridine for 15 min and the cytoplasmic RNA was isolated (*Materials and Methods*). 3.3 A_{260} units of purified cytoplasmic RNA containing about 6000 cpm of ^{3}H-uridine labeled RNA was incubated in 1 ml 0.3 *M* NaCl, 0.01 *M* Tris pH 7.4, and 2 mM $MgCl_2$ with 50 μg RNase and 50 μg DNase for 30 min at room temperature. Subsequently SDS was added to 0.5% and EDTA to 0.01 *M*. The solution was then incubated with 100 μg pronase for 30 min at room temperature and chromatographed on a 6% agarose column after the addition of phenolred as a tracking dye.[2] Ten-minute fractions (vol = 6 ml) were collected. The transmission at 253 mμ (—) was recorded by a UVICORD (LKB Instruments, Uppsala, Sweden). TCA-precipitable radioactivity (●) was determined as described in Fig. 1.

(B) Agarose chromatography of the cytoplasmic RNA of virus-infected control cultures which were prepared as in Figure 4*A* but actinomycin D was omitted. 1.4 A_{260} units of purified cytoplasmic RNA, containing about 200,000 cpm of ^{3}H-uridine labeled RNA (●) was digested with nucleases and further treated as described in Figure 4*A*.

DNA by two different methods further supports the previous conclusion[2] that it is double-stranded RNA and not a DNA-RNA hybrid. The double-stranded RNA appears to exist in the cytoplasm of infected cells in forms of partially double-stranded RNA's with a sedimentation coefficient higher than that of the double-stranded core. The kinetics of the appearance of the double-stranded RNA suggest that it is a "late" product of viral intracellular biosynthesis. Less

TABLE 3. *Inhibition of the biosynthesis of single- and double-stranded vaccinia-virus RNA by actinomycin D.*

Concentration of actinomycin D in medium	Total ^{3}H-uridine incorporated per A_{260} unit of cytoplasmia RNA	^{3}H-uridine incorporated into RNase-resistant RNA per A_{260} unit of cytoplasmic RNA
None	140,000 cpm	1,120 cpm
10 μg/ml	1,730 cpm	48 cpm

Vaccinia-virus infected cells were labeled with ^{3}H-uridine for 15 min. The cytoplasmic RNA was then isolated (*Materials and Methods*) and the total TCA-precipitable radioactivity was determined (see Fig. 1) from an appropriate aliquot. The RNase-resistant RNA was determined from other aliquot after nuclease digestion and chromatography on a 6% agarose column (see text).

than 0.001 per cent of the double-stranded RNA of virus-infected cells could be detected in virus purified from the medium 20 hours after infection.[8]

The synthesis of the RNase-resistant RNA of vaccinia virus-infected cells was inhibited by actinomycin D under different experimental conditions. This could mean that most of the RNase-resistant RNA that is made in the absence of actinomycin D is directly transcribed from viral DNA, but other explanations are possible. Because too little RNase-resistant RNA was made in virus-infected cells in the presence of actinomycin D, its origin from viral or cellular DNA has not been determined. Its synthesis could result from the transcription of RNA or of DNA-RNA hybrids by DNA-dependent RNA polymerase of virus-infected cells. The former may be analogous to the ability of bacterial DNA-dependent RNA polymerase to catalyze RNA-dependent RNA synthesis.[9]

The synthesis of double-stranded RNA in several uninfected animal cells has recently been described.[2, 10] The synthesis of this cellular double-stranded RNA was also found to be less sensitive to actinomycin D than was that of cellular single-stranded RNA,[10] but the question whether both the vaccinia virus-specific and the animal cell-specific double-stranded RNA's are made by similar mechanisms or have similar biological functions cannot be answered on the basis of the available evidence.

Bøvre and Szybalski have recently reported the synthesis of complimentary RNA corresponding to overlapping segments of the b2 region of coliphage λ.[12] The complimentary RNA can be converted to double-stranded RNA by annealing. Our findings that the vaccinia virus double-stranded RNA arises via a DNA-dependent mechanism and that it exists in the cytoplasm of infected cells as double-stranded cores with single-stranded tails is consistent with the pattern of convergent transcription described Bøvre and Szybalski.

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