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Evidence for the Presence of RNA in the Purified Virions of Vaccinia Virus

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Vaccinia virus, strain WR, was propagated in HeLa cells, L mouse fibroblasts, or primary chicken embryo fibroblasts in the presence of [5-^{3}H]uridine. Carefully purified virions were found to contain significant amounts of labeled trichloroacetic acid-precipitable material which was rendered acid soluble when digested with pancreatic RNase or hydrolyzed in alkali. Controlled degradation of virions with Nonidet P-40 and 2-mercaptoethanol demonstrated that 65 to 80% of the [5-^{3}H]uridine-labeled molecules resided in the viral core. When the total nucleic acids were extracted from viral cores prepared from virions propagated in HeLa cells, 30 to 50% of the total incorporated [5-^{3}H]uridine was found in RNA; in L mouse fibroblasts, 40 to 50%; in primary chicken embryo fibroblasts, 50 to 60%. The RNA molecules do not appear to be covalently linked to the viral DNA genome but sediment in sodium dodecyl sulfate-sucrose gradients as 8 to 10S species relative to ribosomal RNA.

The composition of purified vaccinia virus has been reported to be about 5% DNA, 2% lipid, 2% phospholipid, and the remainder protein (9, 30, 36). Careful analysis of [^{14}C]uridine-labeled virions has demonstrated that 0.1 to 0.2% (by weight) of the total virus particle could be recovered as RNA even after treatment of purified virions with ribonuclease (26). We undertook a study of the nucleic acids associated with purified virions for two reasons. First, it has been reported that RNA has a role in DNA replication (7, 18, 32, 34), and ribonucleotides covalently bound to DNA in T4 bacteriophage have been detected (31). The possibility that a similar role for RNA in the replication of poxvirus DNA seemed reasonable. Secondly, we have observed the generation of DNA fragments when DNA from mature virions was incubated in alkali (J. A. Holowczak and W. Fill, unpublished observations). Ribonucleotide sequences distributed throughout the DNA genome could be one explanation for such instability in alkali.

HeLa S3 cells and L mouse fibroblasts were grown in spinner culture (1, 22). Primary chicken embryo fibroblasts were prepared from 8- to 9-day-old embryos by trypsinization, and the cells were seeded in 32-ounze (ca. 0.946 liter) Brockway bottles in Eagle medium (5). The WR strain of vaccinia virus was used throughout these studies. A stock preparation containing 2×10^{11} elementary bodies per ml with a PFU/elementary body ratio of 1:20 was employed. In all experiments cells were infected at an input multiplicity of 500 elementary bodies per cell. At 1 h after infection, [5-^{3}H]uridine (27.9 Ci/mmol) or [2-^{14}C]thymidine (56 mCi/mmol) (New England Nuclear) was added, and 24 h later the cells were harvested and frozen. Virions were purified (11, 12) and in some experiments centrifuged to equilibrium in 20 to 50% (wt/vol) potassium tartrate gradients prepared in 0.001 M phosphate buffer (pH7.2) or in 20 to 60% (wt/wt) sucrose gradients prepared in D_2O containing 0.001 M phosphate buffer, pH 7.2. Purified virions were treated with RNase (100 μg/ml, 30 min, room temperature), collected by centrifugation, and dispersed in buffer with the aid of sonic treatment. Portions of the virus preparations before and after RNase treatment were precipitated with trichloroacetic acid, the precipitates were collected on membrane filters (Millipore Corp.) and dried, and the radioactivity present was determined by liquid scintillation spectrometry. Virions were sequentially degraded with Nonidet P-40, and the core fraction was isolated as described previously (6, 10). Total nucleic acids were isolated from viral cores by the method of Kates and Beeson (15). Isopycnic centrifugation of the purified nucleic acids was carried out in CsCl (33). Portions from gradient fractions or in some cases alternate fractions were treated with RNase (100 μg/ml, 37 C 1 h) and digested with 0.2 N NaOH (18 h, 37 C) or

0.3 N NaOH (100 C, 60 min) to determine the distribution of RNA in the gradients.

Vaccinia virions propagated in HeLa cells, L mouse fibroblasts, or chicken embryo fibroblasts were purified as described above and banded in 20 to 40% sucrose gradients until the specfic activity (counts per min per OD 260 nm units) of the viral preparation became constant. When virions propagated in L mouse fibroblasts or HeLa cells were analyzed by equilibrium centrifugation in potassium tartrate gradients, greater than 90% of the [5-^{3}H]uridine radioactivity remained associated with the virions in a trichloroacetic acid-precipitable form. In preparations derived from chicken embryo fibroblasts, 15 to 25% of the total [5-^{3}H]uridine radioactivity which sedimented with virions in sucrose gradients now banded at the top of the potassium tartrate gradients, separate from the major virion peak. Virions were sequentially degraded with Nonidet P-40 and 2-mercaptoethanol, and the products of the degradation were analyzed in 20 to 60% sucrose gradients prepared in D_2O. It could be demonstrated that under these conditions of analysis [5-^{3}H]uridine radioactivity remained associated with virions, virions treated with Nonidet P-40, and with viral cores. The cores prepared and analyzed under these conditions had a density of 1.29 g/cm^3, virions 1.24 g/cm^3.

Virions labeled with [5-^{3}H]uridine propagated in a variety of host cells were analyzed to determine what kinds of macromolecules were labeled and where they resided in the virion (Table 1). When complete virions were treated with RNase or the outer layers of the virions were removed by detergent treatment in the presence of 2-mercaptoethanol, 20 to 40% of the [5-^{3}H]uridine radioactivity associated with the purified virions was removed. This suggests that RNA molecules, tightly bound to the surface of the virions, were present. Consistent with the results of analysis in potassium tartrate gradients, virions prepared in chicken embryo fibro-

TABLE 1. *Distribution and nature of [5-^{3}H]uridine-labeled macromolecules in vaccinia virions*

Host cell and radioactive label employed[a]	Sp act (counts/min/μg of DNA)[b]	Total counts/min removed from virions by RNase (%)[c]	Total counts/min removed by treatment with NP-40 and 2-mercaptoethanol (%)[d]	Total counts/min recovered in viral cores (%)[d]	Total label[e] (%) in nucleic acids (prepared from cores) of:	
					RNA	DNA
L-Mouse fibrolasts ([5-^{3}H]uridine)	810	10–12	23 (50)	74	42–50	50–60
S-3 HeLa cells ([5-^{3}H] uridine)	425	10–15	28 (30)	72	34–43	55–60
Chick embryo fibroblasts ([5-^{3}H]uridine)	716	10–24	36(87)	64	52–64	36–50
S-3 HeLa cells ([2-^{14}C] thymidine)	27,000	<2	10 (0)	89	<3	97

[a] HeLa S3 cells and L mouse fibroblasts were propagated in spinner culture. Chicken embryo fibroblasts were primary cultures grown as monolayers. Cells were infected, and the appropriate label was added (2 μCi of [5-^{3}H]uridine per ml or 0.1 μCi of [2-^{14}C]thymidine per ml). After 24 h the cells were harvested, collected by centrifugation, and frozen.

[b] Virions were purified as described in the text. Portions of each preparation were removed and trichloroacetic acid-precipitable radioactivity was determined. The nucleic acid content for each preparation was estimated using a spectrophotometric method (29).

[c] Purified virions were treated with RNase (100 μg/ml, 30 min, room temperature) as described in the text. The range in values reflect determinations carried out with three separate virus preparations.

[d] Virions were sequentially degraded as described previously (6, 10, 11). The figures in parentheses indicate the percentage of the radioactivity released from the virions by treatment with Nonidet P-40 (NP-40) and 2-mercaptoethanol which were rendered acid soluble after RNase treatment (100 μg/ml; 1 h, 37 C).

[e] Cores were prepared as described in footnote *d*, and nucleic acids were extracted as described in the text. Portions of the ethanol-precipitated nucleic acids were collected by centrifugation, dissolved in 10^{-2} M Tris buffer (pH 7.5), and treated with RNase (100 μg/ml) or DNase (100 μg/ml in the presence of 0.01 M $MgCl_2$) for 1 h at 37 C. Samples not treated with enzymes and those treated with nucleases were precipitated with trichloroacetic acid, and the amount of [5-^{3}H]uridine incorporated into RNA or DNA was estimated from the amount of radioactivity rendered acid soluble after treatment with the appropriate enzyme.

blasts were found to have the largest amount of [5-^{3}H]uridine-labeled molecules on their surface. Some of these molecules were not as tightly associated with the virion structure as those on virions prepared in L cells or HeLa cells and were partially released when virions were exposed to potassium tartrate. Part of the radioactivity released by detergent treatment remained acid insoluble after RNase treatment, suggesting that DNA molecules were also present on the surface of the virions. Evidence for DNA molecules which sedimented at about 27*S* in neutral and alkaline sucrose gradients on the surface of partially purified poxvirus preparations has recently been reported (24). Vaccinia cores contain endogeneous enzymes which can actively synthesize mRNA and such molecules are released from the core (15, 16). The possibility that during degradation of virions some RNA was lost from the cores cannot be excluded. The fact that more radioactivity was removed by detergent and 2-mercaptoethanol treatment than by RNase digestion of complete virions (Table 1) supports this possibility. The amount of RNA actually in the core, therefore, may be greater than the results indicate. Alternatively, some RNA molecules may actually reside beneath the surface of the virion but outside the core.

As indicated in Table 1, depending upon the host cell used to propagate the virus, 40 to 60% of the [5-^{3}H]uridine radioactivity in purified nucleic acids isolated from viral cores was rendered acid soluble after treatment with RNase or alkali digestion. These results demonstrated that polyribonucleotides were present in purified virions and may be in close association with the viral genome.

To determine if the RNA sequences present in the viral cores were covalently linked to the DNA genome or existed as species separate from the genome, three kinds of experiments were carried out. First, cores were lysed with detergent essentially as described by Sarov and Becker (29) and Parkhurst et al. (24). The released nucleic acids were then analyzed in 5 to 20% linear sucrose gradients. The RNA present in the core, as detected by its sensitivity to hydrolysis in alkali, sedimented as a peak near the top of the gradient, distinct from the viral DNA genome which sedimented at about 72*S*.

Next, total nucleic acids were extracted and purified from cores as described above and analyzed by isopycnic centrifugation in CsCl. [5-^{3}H]uridine material was recovered at a density of 1.695 g/cm^3. Poxvirus DNA has previously been reported to have a buoyant density of 1.695 g/cm^3 (33). The density of the nucleic acid was not altered by treatment with RNase, nor was there a significant loss of radioactivity from the 1.695 g/cm^3 peak when it was digested with RNase.

Finally, when the nucleic acids purified from viral cores were analyzed in 15 to 30% sucrose-sodium dodecyl sulfate gradients the results shown in Fig. 1 were obtained. A major peak of material sedimenting at about 20 to 25*S* and material sedimenting at 8 to 10*S* were detected in each case. The 20 to 25*S* material was stable in alkali but was rendered acid soluble after DNase treatment and therefore was viral DNA. The material sedimenting at 8 to 10*S* appeared to contain some DNA sequences, but more than 90% of the [5-^{3}H]uridine radioactivity in this

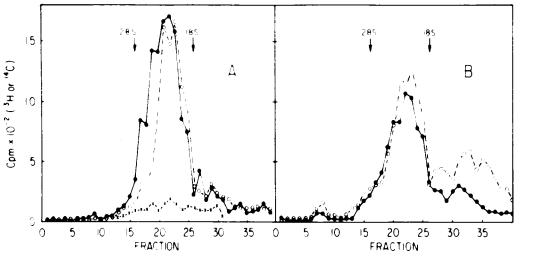

FIG. 1. *Sedimentation analysis of purified nucleic acids prepared from viral cores labeled with [2-^{14}C]thymidine or [5-^{3}H]uridine in sucrose sodium dodecyl sulfate gradients. Total nucleic acids were extracted and purified from viral cores. Nucleic acids precipitated with ethanol were collected by centrifugation and dissolved in sodium dodecyl sulfate buffer (0.1 M NaCl, 0.001 M EDTA, 0.01 M Tris-hydrochloride, 0.5% sodium dodecyl sulfate, pH 7.4). Sedimentation markers of 28S and 18S RNA were prepared from ribosomes isolated from HeLa cells. Samples were loaded onto 16-ml 15 to 30% (wt/wt) sucrose gradients prepared in sodium dodecyl sulfate buffer and centrifuged for 18 h; (24,000 rpm at 23 C) in a Spinco SW27.1 rotor. Duplicate gradients were analyzed for each sample. Fractions of one gradient were precipitated with trichloroacetic acid immediately; to fractions of the duplicate gradient 0.3 N NaOH was added, and the gradient was incubated at 100 C for 60 min and then precipitated with trichloroacetic acid. The distribution of radioactivity from treated and untreated gradients has been plotted on the same graph. The sedimentation of 28S and 18S RNA markers, detected by their absorption at 260 nm, was identical in each gradient. (A) [2-^{14}C]Thymidine-labeled nucleic acids; (B) [5-^{3}H]uridine-labeled nucleic acids from virions propagated in L cells or HeLa cells. Symbols: O, total trichloroacetic acid-precipitable radioactivity; ●, trichloroacetic acid-precipitable radioactivity after alkaline hydrolysis (0.3 N NaOH, 60 min, 100 C); ×, trichloroacetic acid-precipitable radioactivity after portions (50 μliters) of gradient fractions were digested with DNase (100 μg/ml, 37 C, 1 h).*

region of the gradient became acid soluble after alkaline hydrolysis.

The total nucleic acids extracted from virions prepared in HeLa cells, chicken embryo fibroblasts, or L mouse fibroblasts were digested with DNase until analysis demonstrated that all remaining trichloroacetic acid-precipitable radioactivity was in the form of polyribonucleotides. This material was then used in the hybridization experiments summarized in Table 2. The RNA recovered from the virions hybridized with viral DNA but not with host cell DNA, demonstrating that the RNA sequences were coded for by the viral genome.

Poxviruses are large, complex animal virions which multiply exclusively in the cell cytoplasm. A number of enzymatic activities have now been detected in purified virions. These include a DNA dependent RNA polymerase (16, 20), deoxyribonucleases (27, 28), a nucleotide phosphohydrolase (8, 21), a protein kinase (17, 25), and a poly A polymerase (3). Whereas the genome of vaccinia virus has been characterized as a single, double-stranded DNA molecule with a molecular weight of 1.6 to 1.8 $\times 10^8$ (2, 13, 29), there existed some experimental evidence for the presence of RNA sequences in purified virions (26). Our results confirm this observation and suggest that the RNA sequences are not covalently linked to the DNA genome but are discrete species which sediment at 8 to 10S relative to rRNA in sodium dodecyl sulfate-sucrose gradients. The RNA isolated from viral cores hybridized specifically with viral DNA and not with the DNA of the host cell in which the virions were propagated. The sedimentation behavior of this RNA is like that reported for "early" RNA sequences transcribed during the viral replication cycle (22).

The inclusion of RNA into mature virions may occur by accident during viral assembly, or the RNA may play a role in viral replication and specific RNA sequences may be required for the virions to be infectious. The demonstration of a new DNase in virus particles grown only in irradiated cells (23) emphasizes the possible accidental integration of cellular enzymes or material coded for by the viral genome into the

TABLE 2. *Hybridization of virion-associated RNA with vaccinia and host cell DNA*

Source of RNA used for hybridization[a]	Source of DNA used for hybridization[b]	Total counts/min used for hybridization[c]	Total counts/min hybridized[d]
Cores from virions grown in HeLa cells	Vaccinia	825	193
		1,853	309
	HeLa cells	1,850	41
Cores from virions grown in L cells	Vaccinia virus	1,750	375
		2,600	750
	L cells	2,600	47
Cores from virions grown in chicken embryo fibroblasts	Vaccinia virus	540	70
		1,080	134
	Chicken embryo fibroblasts	1,080	37

[a] [5-^{3}H]Uridine-labeled virions were purified, and cores were prepared by treating the virions with Nonidet P-40 and 2-mercaptoethanol. Nucleic acids were extracted and purified as described by Kates and Beeson (15). The nucleic acids were digested with DNase (50 μg/ml in presence of 0.01 M $MgCl_2$) which had been treated with iodoacetate (35). Digestion was followed by removing 10-μliter portions and precipitating with trichloroacetic acid. When the reaction was completed, rRNA was added as a carrier, and the RNA in the digests was purified as described by Oda and Joklik (22).

[b] DNA from virions was purified as described above but included digestion with pancreatic RNase (50 μg/ml, 1 h, 37 C). Host cell DNA was isolated from nuclei prepared by lysis of cells in Triton X-100 (19). DNA was denatured with alkali treatment at pH 12.5, and filters with 1 μg of denatured DNA were prepared for hybridization.

[c] RNA was dissolved in 4 × SSC (0.15 M NaCl plus 0.015 M sodium citrate). More than 95% of the total counts per minute in each preparation was rendered acid solubleby RNase (100 μg/ml, 1 h, 37 C).

[d] Hybridization reactions were carried out as described by Oda and Joklik (22). Blanks for the reactions averaged 34.0 counts/min.

structure of the virion. It will be necessary to examine virions of other members of the poxvirus group to determine if inclusion of polyribonucleotides into mature virions is a general phenomenon. We are now carrying out such experiments and competitive hybridization studies to determine if the RNA associated with virions contains "early" or "late" viral mRNA sequences.

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