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Ribonucleotides Linked to DNA of Herpes Simplex Virus Type 1

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Cells of a continuous cell line derived from rabbit embryo fibroblasts were infected with herpes simplex type 1 virus (HSV-1) and maintained in the presence of either [5-^{3}H]uridine or [*methyl*-^{3}H]thymidine or $^{32}PO_4^{3-}$. Nucleocapsids were isolated from the cytoplasmic fraction, partially purified, and treated with DNase and RNase. From the pelleted nucleocapsids, DNA was extracted and purified by centrifugation in sucrose and cesium sulfate gradients. The acid-precipitable radioactivity of [5-^{3}H]uridine-labeled DNA was partially susceptible to pancreatic RNase and alkaline treatment; the susceptibility to the enzyme decreased with increasing salt concentration. No drop of activity of DNA labeled with [^{3}H]thymidine was observed either after RNase or alkali treatment. Base composition analysis of [5-^{3}H]uridine-labeled DNA showed that the radioactivity was recovered as uracil and cytosine. In the cesium sulfate gradient, the purified [5-^{3}H]uridine-labeled DNA banded at the same position as the ^{32}P-labeled DNA. The present data tend to suggest that ribonucleotide sequences are present in HSV DNA, that they are covalently attached to the viral DNA, and that they can form double-stranded structures.

It is accepted that the genome of herpes simplex virus (HSV) consists of double-stranded DNA (3, 5, 18). The presence of unusual bases has not been reported (3, 9, 12). Recently, the presence of RNA in the genetic material of bacteriophages T4 (19) and T5 (16) was demonstrated. In the latter paper the possible relationship between the presence of RNA stretches in the DNA backbone and DNA fragmentation under alkaline conditions (1) was stressed. With respect to the existence of alkali-labile sites in HSV DNA (6-8), the question has been raised whether the genetic material of HSV does not contain some ribonucleotide sequences. The present paper describes experiments, the results of which seem to indicate that ribonucleotides are really linked to HSV DNA.

MATERIALS AND METHODS

Virus. HSV type 1 (HSV-1) virus, strain Kupka (isolated by R. Benda), was used in the present experiments. The virus was grown in rabbit embryo fibroblast (REF) cells (17).

Chemicals. Pancreatic DNase (RNase-free) was purchased from Worthington Biochemical Corp. Pancreatic RNase (five times crystallized, A grade) was obtained from Calbiochem; it was heated for 10 min at 80 C before each experiment. [5-^{3}H]uridine (24.2 Ci/mmol) and [*methyl*-^{3}H]thymidine (19.37 Ci/mmol) were obtained from the Institute for Research, Production and Use of Radioisotopes (ÚVVVR), Prague, and $^{32}PO_4^{3-}$ was from Isocomerz GMB, Berlin (GDR).

Solutions. Virus buffer consisted of 0.15 M NaCl and 0.02 M Tris-hydrochloride, pH 7.5. Neutral DNA buffer consisted of 1 M NaCl, 0.001 M EDTA, and 0.05 M Tris-hydrochloride, pH 7.5. Reticulocyte standard buffer (RSB) contained 0.01 M NaCl, 0.001 M $MgCl_2$, and 0.01 M Tris-hydrochloride, pH 7.5. Standard saline citrate (1× SSC) consisted of 0.15 M NaCl and 0.015 M trisodium citrate.

Preparation of nucleocapsids. The method of Kieff et al. (8) was used to prepare nucleocapsids and DNA for velocity sedimentation. Medium was drained from confluent monolayers grown up in 1,200-ml Roux bottles. A 10-ml amount of virus suspension was added to each culture to reach a multiplity of infection of 10 PFU per cell. Adsorption was carried out at 37 C for 2 h, during which time the inoculum was spread by rocking the bottles at 20-min intervals. Inoculum was then drained, monolayers were washed, and 70 ml of Eagle minimal essential medium (MEM) supplemented with 2% dialyzed calf serum was added to each bottle. The cultures were then placed at 37 C. They were incubated from 4.5 to 22 h postinfection either with 10 μCi of [*methyl*-^{3}H]thymidine per ml or 10 μCi of [5-^{3}H]uridine, or with 9 μCi of $^{32}PO_4^{3-}$ per ml. The labeling with ^{32}P was done in phosphate-free MEM. Cells were harvested at 22 h by scraping, and were centrifuged at 800 × *g* for 10 min. The harvest was comprised of approximately 2×10^6 cells in the case of [^{3}H]uridine labeling, of approximately 4×10^7 cells in the case of $^{32}PO_4^{3-}$ labeling, and of approximately 4×10^7 cells in the case of [^{3}H]thymidine

labeling. The cell pellet was washed with phosphate-buffered saline (PBS), resuspended in RSB containing 0.5% Nonidet P-40 (Shell Chemical Co., New York, N.Y.), and left for 10 min at 4 C. The cells were then homogenized with four strokes of a tight-fitting Dounce homogenizer, and the nuclei were removed by centrifugation at 800 × *g* for 10 min at 4 C. A 5-ml amount of the supernatant containing viral nucleocapsids was layered onto 57-ml linear sucrose density gradients (10 to 50%, wt/wt) made in virus buffer and was centrifuged for 1 h in the MSE rotor (3 by 65 ml) in the MSE 65 Superspeed centrifuge at 23,000 rpm at 4 C. After centrifugation, approximately 2.4-ml fractions were collected through the bottom of centrifugation tubes. A portion (10 μliters) of each fraction was deposited on Whatman no. 3 paper, dried, washed twice with 5% trichloroacetic acid and once with 96% ethanol. The radioactivity was measured for 10 min in a Packard Tricarb scintillation spectrometer by using scintillation solution containing 4.9 g of 2,5-diphenyloxazole and 0.1 g of 1,4-bis-(2,5-phenyloxazolyl)-benzene per liter of toluene. Fractions containing the peak activities were pooled, mixed with 4 volumes of RSB, and treated with DNase (20 μg/ml) and RNase (50 μg/ml) for 1 h at 37 C. This material was centrifuged for 2 h in the MSE rotor (3 by 65 ml) at 23,000 rpm at 4 C. The sediment was resuspended in virus buffer and the material was spun down for 90 min under the same conditions.

DNA extraction and purification. The pelleted nucleocapsids were dissolved in neutral DNA buffer containing 0.5% (wt/vol) sodium dodecyl sulfate (Sigma) and 2% (wt/vol) sodium *N*-lauryl sarcosinate (Koch and Light, Ltd.) and were gently rolled with phenol for 2 min at 60 C. The phenol phase was removed by centrifugation and the aqueous phase was rolled with chloroform-isoamylalcohol (2%, vol/vol) until clear. This sample was gently layered onto the top of a 11.3-ml sucrose gradient (10 to 30%, wt/wt) made in neutral DNA buffer and was centrifuged for 3.5 h in an SW 41 rotor of Spinco L2-65B centrifuge at 40,000 rpm at 20 C. Fractions (0.55 ml) were collected from the top. The activity in each fraction was measured as described above. The fractions containing the peak activities were pooled and dialyzed four times for 20 h against 1,000 volumes of 0.1× SSC. Thereafter, each DNA sample (approximately 1 ml) was mixed with 2.6 ml of Cs_2SO_4-saturated water solution and with Tris-EDTA solution to give a final concentration of 0.001 M EDTA and 0.01 M Tris-hydrochloride, pH 7.4, and a final volume of 6 ml. The materials were placed in polypropylene centrifugation tubes with paraffin oil to fill the tubes. The tubes were centrifuged for 72 h in a rotor MSE (8 by 14 ml) in MSE 65 Superspeed centrifuge at 50,000 rpm at 20 C. Fractions of 20 drops each were collected from the bottom of the tubes and were mixed with equal amounts of 0.1× SSC. The radioactivity in each fraction was measured as described. The fractions containing the peak activities were pooled and dialyzed the same as the sucrose gradient centrifugation. The optical density at 260 nm of these fractions was determined.

Base composition analysis. The method of Bendich (2) was used for total acid hydrolysis of DNA and subsequent paper chromatography. The 0.5-ml portions of [^{3}H]uridine or [^{3}H]thymidine-labeled samples were dried under vacuum; 1 mg of carrier DNA (Salmon sperm, A grade, Calbiochem) and 0.5 ml of 88% formic acid were added. Pyrex glass tubes containing this solution were sealed and heated for 30 min at 175 C. Samples of [^{3}H]uracil, [^{3}H]thymine, and [^{3}H]cytosine (ÚVVVR, Prague) used as standards for paper chromatography were treated in the same way. The treated samples were dried under vacuum above NaOH, diluted in one drop of 1 N HCl, and chromatographed on Whatman no. 3 paper with a solvent consisting of isopropanol:HCl:water (65:16.7:18.3). The position of bases was detected by examination under UV light. The chromatograms were cut into strips, and the radioactivities of all samples were determined.

Isopycnic centrifugation of purified native and denatured DNA in Cs_2SO_4. Two samples consisting of mixtures of [^{3}H]uridine-labeled HSV DNA and of ^{32}P-labeled HSV DNA were analyzed. Prior to centrifugation, the samples were mixed with 0.1 mg of sonic-treated calf thymus DNA (Koch and Light, Ltd.). One of the samples was heated at 100 C for 6 min and dipped quickly in an ice bath. Thereafter, 0.8-ml volumes of each DNA sample were mixed with 3 ml of Cs_2SO_4-saturated water solution and with Tris-EDTA solution to give a final volume of 6 ml. The materials were centrifugated as described. Fractions of 15 drops each were collected from the bottom of the tubes. The radioactivity was measured directly after mixing each sample with 1.3 ml of water and 5 ml of Insta-Gel (Packard).

RESULTS

Labeling, extraction, and purification of HSV DNA. [5-^{3}H]uridine used in this study can either be converted to deoxycytidine triphosphate that is incorporated into the DNA or can be incorporated into RNA as uridylic and cytidylic acids. Besides uridine, we used [*methyl*-^{3}H]thymidine for specific labeling of DNA and $^{32}PO_4^{3-}$ for labeling DNA and the possibly present RNA stretches. Cytoplasmic extracts of HSV-infected REF cells formed one band in the density sucrose gradient (Fig. 1). The position of the radioactive peak was the same in each case of labeling and corresponded to the bottom band of UV-absorbing material containing predominantly full nucleocapsids (8; Hirsch, unpublished data). The fractions containing the peak activities were pooled and treated with DNase and RNase.

Nucleic acid was extracted from the pelleted nucleocapsids and fractionated in neutral sucrose gradients (Fig. 2, A, B, C). The fractions containing the peak activities were pooled and centrifuged in caesium sulphate gradients (Fig. 2, D, E, F). The peak activity-containing fractions were pooled and used as the starting mate-

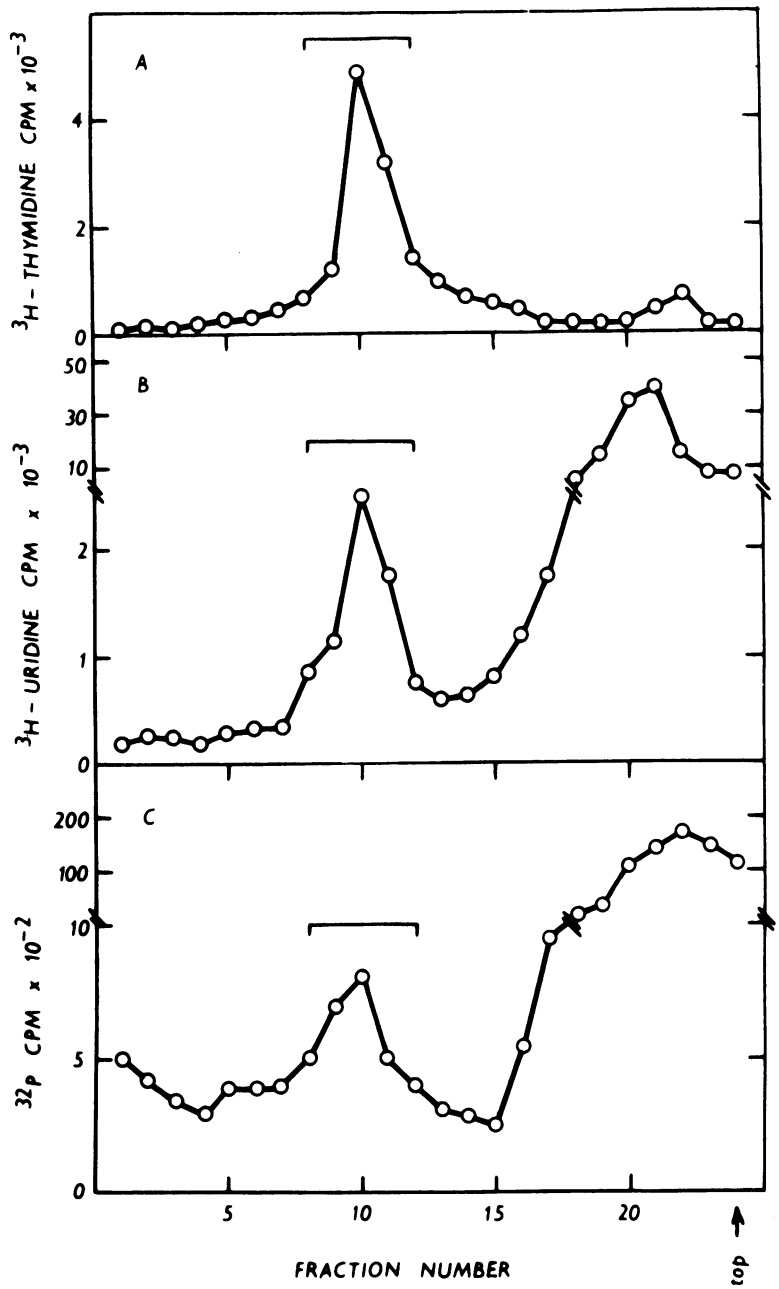

FIG. 1. *Sedimentation of nucleocapsids in neutral sucrose density gradients. For labeling and extraction of nucleocapsids and for gradient preparation see Materials and Methods. The direction of centrifugation is from right to left. The bars indicate fractions that were pooled for HSV-DNA isolation. (A) [^3H]thymidine-labeled nucleocapsids isolation; (B) [^3H]uridine-labeled nucleocapsids; (C) ^{32}P-labeled nucleocapsids.*

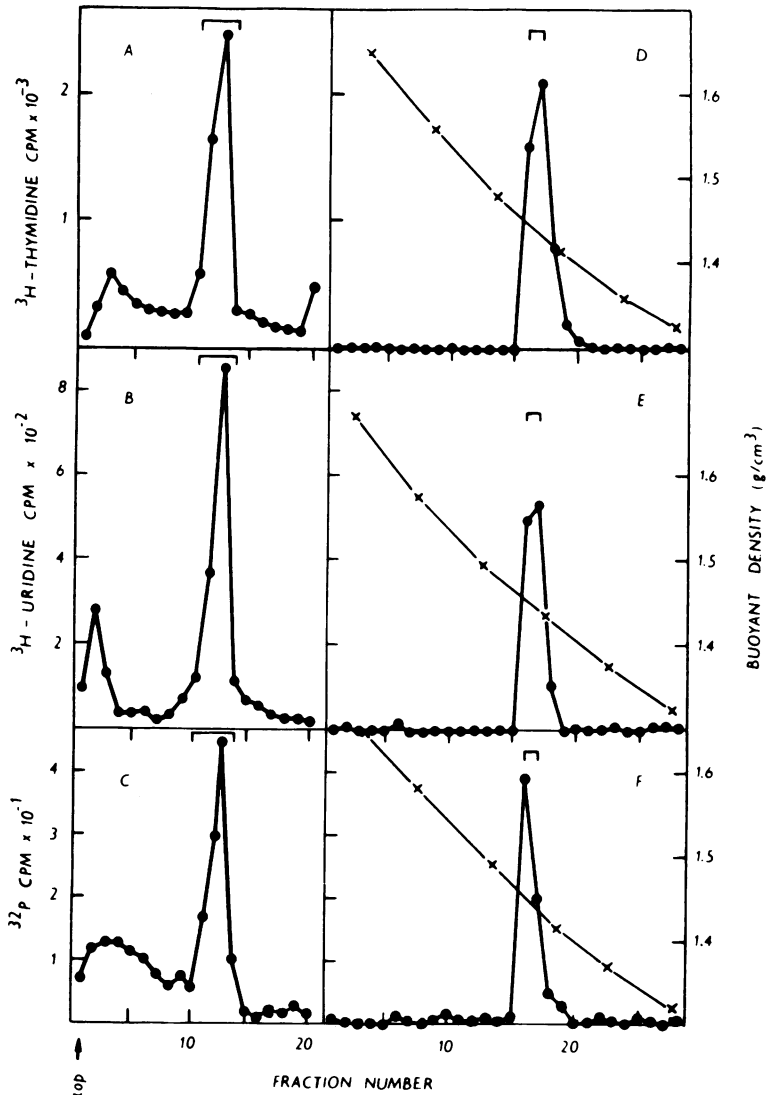

FIG. 2. *Purification of HSV DNA by centrifugation in neutral sucrose density gradients and in cesium sulfate gradients. For DNA labeling and extraction and for gradient preparation see Material and Methods. The direction of centrifugation in the case of sucrose density gradients is from left to right. The bars indicate fractions that were pooled and used for further work. (A) [^3H]thymidine-labeled HSV DNA in neutral sucrose gradient, (B) [^3H]uridine-labeled HSV DNA in neutral sucrose gradient; (C) ^{32}P-labeled HSV DNA in neutral sucrose gradient; (D) [^3H]thymidine-labeled HSV DNA in cesium sulfate gradient, (E) [^3H]uridine-labeled HSV DNA in cesium sulfate gradient; (F) ^{32}P-labeled HSV DNA in cesium sulfate gradient. Symbol ×, buoyant density.*

rial for all further experiments.

Association of ribonucleotide sequences with extracted DNA. Table 1 shows the RNase, DNase, and alkali susceptibility of the [^{3}H]uridine or the ^{32}P-labeled HSV DNA. There was 25 and 20% decrease of [^{3}H]uridine activity after treatment with pancreatic RNase and alkali, respectively, whereas about 84% was lost after treatment with pancreatic DNase. The ^{32}P-labeled DNA was susceptible to DNase; the decrease of the radioactivity after RNase treatment was less than 5%. No decrease of acid-insoluble activity was observed after either RNase or alkali treatment when the DNA was prelabeled with thymidine.

The dependence of RNase sensitivity of uridine-labeled HSV DNA on salt concentration is shown in Table 2. Susceptibility decreased with increasing salt concentration.

Base composition analysis of the [5-^{3}H]uri-

TABLE 1. *Nuclease and alkali susceptibility of [^3H]uridine or ^{32}P-labeled HSV DNA*[a]

Treatment	Radioactivity retained (counts/min)		Radioactivity retained (%)	
	[5-^{3}H] uridine	^{32}P	[5-^{3}H] uridine	^{32}P
None	408	40	100.0	100.0
DNase	65	6	16.0	15.2
RNase	306	38	75.2	95.8
KOH	327	38	80.2	95.0

[a] Portions of [^{3}H]uridine-labeled DNA were mixed with ^{32}P-labeled DNA. Triplicate 0.2-ml portions in 15 mM NaCl, 5 mM Tris-hydrochloride, pH 7.4, and 0.5 mM trisodium citrate were then exposed to DNase (30 μg/ml, 37 C, 60 min), RNase (50 μg/ml, 37 C, 60 min), or KOH (0.2 N, 100 C, 20 min). In the case of DNase treatment, the reaction mixture contained 6 mM $MgCl_2$. Enzyme-treated specimens were acidified with trichloroacetic acid to the final concentration of 5%. After 30 min of standing in an ice bath the solution was filtered through HAWP Millipore filters, washed with 5% trichloroacetic acid and with 96% ethanol, dried, and counted for 10 min with a toluene scintillator fluid as described in Materials and Methods. The samples treated with KOH were neutralized with 1 N HCl, and then trichloroacetic acid was added. Specific activity of [5-^{3}H]uridine-labeled and ^{32}P-labeled HSV DNA was 3×10^3 counts per min per μg and 2×10^3 counts per min per μg, respectively.

TABLE 2. *Dependence of RNase sensitivity of uridine-labeled HSV-1 DNA on salt concentration*[a]

Salt concn	Radioactivity retained	
	Counts/min	%
Control (untreated in 0.1× SSC)	410	100.0
RNase treatment:		
0.1× SSC	312	76.1
0.2× SSC	328	80.0
0.5× SSC	358	87.2
1.0× SSC	357	87.2
2.0× SSC	383	93.3

[a] Duplicate 0.1-ml portions of [^{3}H]uridine-labeled DNA were diluted in different SSC concentration and treated with RNase at a final concentration of 50 μg/ml at 37 C for 60 min. For determination of acid-precipitable radioactivity and for specific activity of DNA see Table 1.

dine-labeled nucleic acid (Fig. 3) showed that the radioactivity was recovered as uracil and cytosine. In a parallel experiment with HSV DNA labeled with [^{3}H]thymidine, the radioactivity was recovered solely as thymine. Whereas 5% of the total activity of uridine-labeled DNA was found in the position of uracil, only 1% of cytosine, used as a standard, was converted to uracil in the course of the hydrolysis procedure used.

Analysis of the nucleic acid in a cesium sulfate gradient resulted in the banding of the [5-^{3}H]uridine-labeled DNA and ^{32}P-labeled DNA at the same position (Fig. 4). This effect was observed for the native as well as for the heat-denatured HSV DNA.

DISCUSSION

The procedure used for the purification of HSV DNA prior to its analysis considerably reduced the possibility of its contamination with RNA of cellular origin. Also, the findings that in cesium sulfate gradients all activities cosedimented and that the peak of [^{3}H]uridine-labeled DNA was susceptible to RNase and alkali treatment provide an important piece of evidence against this possibility. Thus, the present data seem to be consistent with the idea that ribonucleotide sequences are linked to HSV DNA.

The molecular state of ribonucleotide sequences associated with HSV DNA is poorly understood at this writing. The cosedimentation of all the activities of the purified heat-denatured DNA in cesium sulfate gradients (Fig. 4) suggests that the ribonucleotide sequences were covalently bound to the viral DNA. (It should be added that RNase treatment of a comparable sample of heat-denatured HSV DNA which had been purified in cesium sulfate gradient resulted in a significant decrease of [^{3}H]uridine-labeled DNA [unpub-

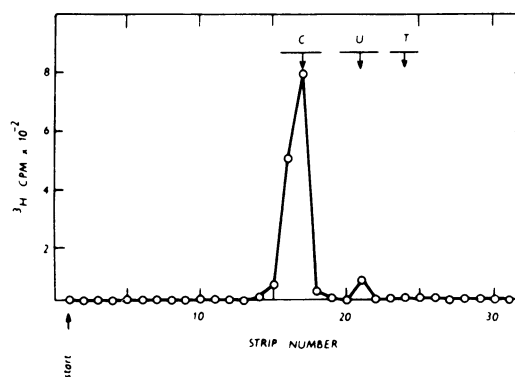

FIG. 3. *Distribution of radioactivity among bases of [^3H]uridine-labeled HSV DNA. For hydrolysis and paper chromatography see Material and Methods. The positions of the individual bases are indicated by lines and the peak of activity of standards by arrows (C, U, and T stand for cytosine, uracil, and thymine, respectively).*

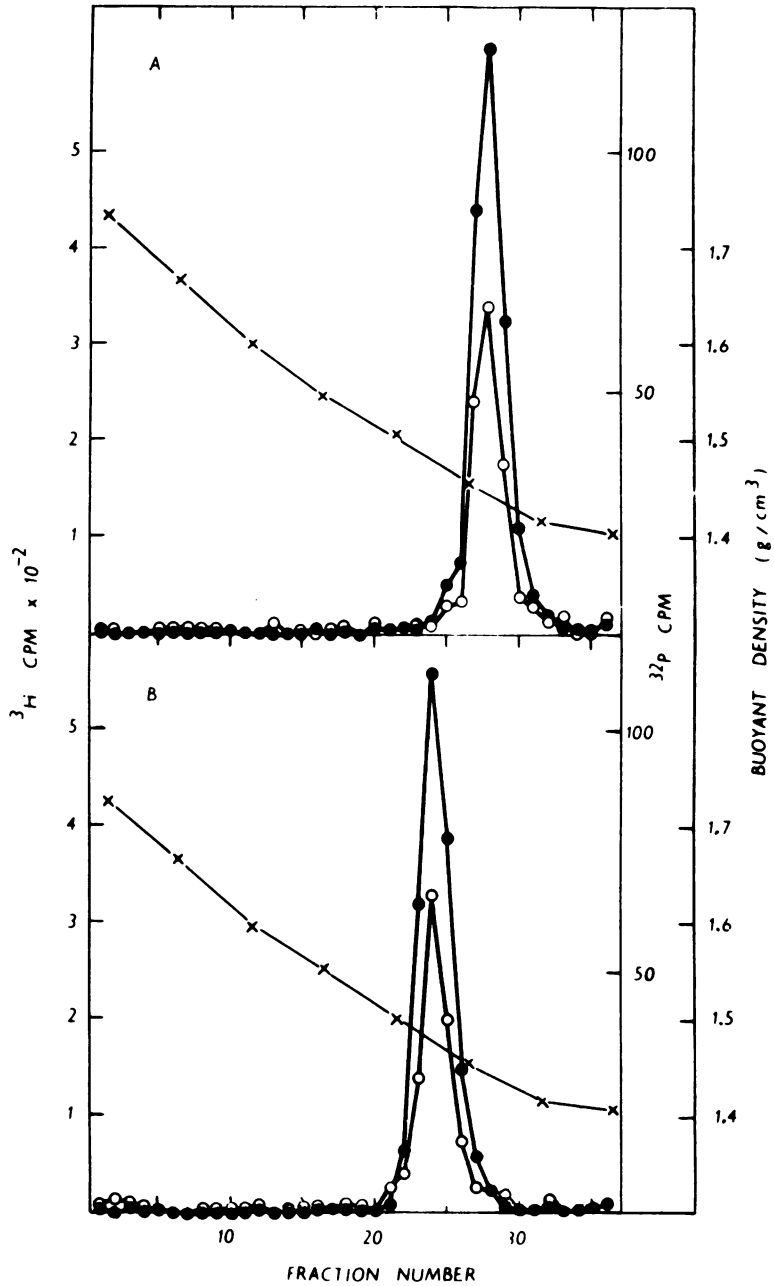

FIG. 4. *Isopycnic centrifugation of [^3H]uridine and ^{32}P-labeled HSV DNA in cesium sulfate. Native HSV DNA (A) and heat-denatured HSV DNA (B) were centrifuged and radioactivity was measured as described in Materials and Methods. Symbols: ●, [^3H]uridine-labeled HSV DNA; ○, ^{32}P-labeled HSV DNA; ×, buoyant density.*

lished observation]). The dependence on salt concentration of the susceptibility of [^{3}H]uridine-labeled DNA to RNase suggests that RNA stretches can form double-stranded, probably hybrid structures. As far as the size of the ribonucleotide segment(s) is concerned, only indirect evidence is available. Although the present results are strongly reminiscent of those reported for T phages (16, 19), the amount of RNase and alkali-sensitive [^{3}H]uridine-labeled

DNA was lower in our preparations than in theirs. This could be due to a more efficient conversion of [5-^{3}H]uridine to cytidine and deoxycytidine in eukaryotic cells; however, it can also be due to a much lower content of ribonucleotides in HSV than in T4 or T5 phages.

The origin and role of the RNA sequences associated with HSV DNA can only be a matter of speculation now. The above discussed results, which suggested that the ribonucleotides were covalently bound to HSV DNA, make it seem improbable that HSV-specific RNA, which could be present in HSV virions, hybridized with the isolated HSV DNA. It seems, therefore, that the RNA sequences were linked to the HSV DNA in the course of the replication process. Since there are many more interruptions per DNA molecule during replication than after maturation is completed (6), it seems probable that RNA stretches, which arise in the replication, are removed only gradually during maturation. They could act as a primer for synthesis of Okazaki pieces (20), but they could also be involved in the process of initiation of HSV-DNA (replicon) replication (4, 10). It is also possible, however, that the presence of RNA stretches within HSV DNA is without any biological significance.

If the present results are confirmed and their interpretation is correct, it would mean that, in addition to animal RNA viruses containing DNA segments (15), animal DNA viruses with RNA sequences also exist. Since a similar pattern of DNA fragmentation as in HSV-1 and -2 viruses (6–8) has been demonstrated in Marek's disease virus (11), Epstein-Barr virus (14), and murine cytomegalovirus (13), it is possible that the presence of RNA stretches within the genetic material is a general phenomenon among the herpesviruses.

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ADDENDUM

After preparing this manuscript we have learned that N. Biswall, B. K. Murray and M. Benyesh-Melnick succeeded in demonstrating the presence of ribonucleotide sequences in HSV type 1 and type 2 DNA (M. Benyesh-Melnick, personal communication).

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