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Evidence for a partial RNA transcript of the small circular component of kinetoplast DNA of Crithidia acanthocephali

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ABSTRACT

The major component of kinetoplast DNA (kDNA) in the protozoan Crithidia acanthocephali is an association of approximately 27,000, 0.8 μm (1.58×10^6 dalton) circular molecules apparently held together in a particular structural configuration by topological interlocking. We have carried out hybridization experiments between kDNA samples containing one or the other of the two complementary (H and L) strands of purified 0.8 μm molecules derived from mechanically disrupted associations and RNA samples prepared either from whole C. acanthocephali cells or from a mitochondrion-enriched fraction. The results of experiments involving cesium sulfate buoyant density centrifugation indicate that whole cell RNA contains a component(s) complementary to all kDNA H strands, but none complementary to kDNA L strands. Similar results were obtained using mitochondrion-associated RNA. Digestion of RNA/DNA hybrids and suitable controls with the single-strand-specific nuclease S1 indicated that 10% of the kDNA H strand is involved in hybrid formation. Visualization of RNA/DNA hybrids stained with bacteriophage T4 gene 32 protein revealed that hybridation involves a single region of each kDNA H strand, equal to approximately 10% of the molecule length. These data suggest that at least 10% of the small circular component of kDNA of Crithidia acanthocephali is transcribed.

INTRODUCTION

A structurally complex DNA, known as kinetoplast DNA (kDNA) is found in the mitochondria of members of the protozoan order kinetoplastida (1). In one such organism, Crithidia acanthocephali, the kDNA comprises on the average 27,000 circular molecules, each with a uniform contour length of 0.8 mμ (1.58×10^6 daltons), which are held together apparently by topological interlocking in a definite ordered manner to form a structure termed an association (2). Long, possibly linear molecules are found associated with some associations. Associations with some species specific variations in structure have been described for kDNA from a variety of species of Trypanosoma (3-7), Crithidia (2), Leishmania (8), and Phytomonas (9). Data obtained from a number of physicochemical and restriction enzyme analyses of C. acanthocephali kDNA are consistent with the view that the small circular component

comprises at least 70% of this DNA, and that while it seems most likely that individual circular molecules have much of their nucleotide sequences in common, nucleotide differences between them do occur (10, 11). Evidence from renaturation studies for a component accounting for approximately 15% of C. acanthocephali kDNA with a sequence complexity of about 18 x 10^6 daltons has been presented (10). Similar conclusions concerning the sequence complexity of the kDNAs of other Crithidia species (12-15) and species of Trypanosoma (16, 17), Leishmania (18-20), and Phytomonas (9) have been reached. Further, it has been shown for some species of the genera mentioned that their kDNA includes a large circular component of molecular weight ranging from 12.2 x 10^6 for T. brucei (17) to 22 x 10^6 for C. luciliae (21).

Recently studies have been carried out to gain information on the transcription product of kDNA. From purified mitochondrial fractions of C. fasciculata, Nichols and Cross (22) reported the isolation of four distinct RNAs, the synthesis of which were inhibited by ethidium bromide. No attempt was made, however, to demonstrate the complementarity of these RNAs to kDNA. However, Simpson and Simpson (23) isolated two distinct RNA molecules of 518 and 1022 nucleotides from purified mitochondrial fractions of L. tarentolae, and convincingly showed that both are complementary to the sequences of the large circular component of this organisms's kDNA and not to sequences of the small circular component. Evidence for RNA present in isolates of whole cell RNA of C. luciliae, which again are complementary to the large circular component of kDNA, but not to the small circular component has been presented by Hoeijmakers and Borst (24).

In this report we present evidence for the occurrence in cells of C. acanthocephali of an RNA molecule which is complementary to approximately 10% of the small circular component of the organisms's kDNA, suggesting that, contrary to the conclusions of Hoeijmakers and Borst (24), this DNA is in fact at least partially transcribed.

MATERIAL AND METHODS

Culture of Organisms. All experiments involved cells of the same strain of Crithidia acanthocephali as that used by Renger and Wolstenholme (2) and originally obtained from Dr. Helene Guttman at the University of Illinois, Chicago Circle. Maintenance of stocks and culture of cells used for DNA and RNA extractions were carried out as described previously (2, 10).

Kinetoplast DNA Isolation. Details of the following techniques were described previously (10). Isolation of whole cell DNA; separation and purifi-

cation of kDNA associations from whole cell DNA; preparation of fractions consisting of 1) covalently-closed, single circular molecules, and 2) mixtures of open single circular molecules and linear molecules of single-circle length (unit-length linear molecules) from kDNA associations; preparation by alkaline cesium chloride centrifugation of heavy (H) and light (L) kDNA strands from fractions consisting of a mixture of open circular and unit-length linear molecules; preparative neutral cesium chloride and ethidium bromide-cesium chloride equilibrium density gradient centrifugation. The molecular forms present in all DNA fractions were verified by electron microscopy (10). The identity and purity of H strand fractions and L strand fractions of kDNA were verified by analytical neutral and alkaline cesium chloride equilibrium density gradient centrifugations (10).

Isolation of Whole Cell RNA. C. acanthocephali was harvested in mid-log phase (≈8 x 10^6 cells/ml) by centrifugation at 6000 x g for 10 min and resuspended in extraction buffer (50 mM sodium chloride, 10 mM sodium acetate, pH 5.1) containing 2mg/ml polyvinylsulfate (PVS) and 2 mg/ml sodium bentonite (prepared by the procedure of Watts and Mathias (25)) at 4°C. Cells were lysed by addition of sufficient 20% sodium dodecyl sulfate (SDS) to yield a final concentration of 2% and whole cell RNA (wcRNA) was isolated by the method of Kirby (26) which included deproteination with redistilled phenol/m-cresol (7:1 w/v), and removal of DNA with pancreatic DNase (RNase free, Worthington). Whole cell RNA was also isolated from C. acanthocephali by pelleting through a two-step cesium chloride gradient following the method of Glisin et al. (27).

Preparation of a Mitochondrion-enriched Fraction. Harvested log phase cells were suspended in a buffer containing 10 mM Tris (pH 7.1), 10 mM potassium chloride, 0.15 mM magnesium chloride, 0.5 M sucrose, 2 mg/ml PVS, and 2 mg/ml sodium bentonite. The cells were disrupted by blending for 20 sec at high speed in a model 5011 Waring blender, and the product centrifuged at 1000 x g for 10 min at 4°C. The pellet was discarded and the supernatant subjected to centrifugation at 1000 x g for 10 min four more times. The final supernatant was centrifuged at 12,000 x g for 10 min and the resulting pellet washed by repeated centrifugation in buffer containing 0.25 M sucrose. The final pellet was layered onto the top of a 10 ml, 1.0 M to 2.2 M linear sucrose (RNase free grade, Sigma) gradient in 50 mM sodium chloride, 10 mM sodium acetate (pH 5.1) and centrifuged at 20,000 rpm for 1 hr at 4° C in a Beckman Spinco Model L2 65B using an SW-41 rotor. The band of mitochondria (at a sucrose molarity of 1.65 M to 1.70 M; ρ = 1.266 g/cm^3 to 1.277 g/cm^3) was

removed using a Pasteur pipet and the RNA (referred to as mitochondrion-associated RNA (mt-assoc RNA)) extracted as described above for wcRNA.

Sucrose Gradient Analyses. Samples of 100 µg to 500 µg of RNA contained in 0.5 ml of extraction buffer were layered onto a 10 ml linear 5% - 20% sucrose gradient (RNase free grade) in 20 mM sodium chloride, 10mM sodium acetate (pH 5.1), and centrifuged at 37,000 rpm for 4.5 or 5 hr at 4°C in an SW-41 rotor. Following centrifugation, 8-drop fractions were collected. Absorbance (260 nm) was determined using a Gilford 2400-S spectrophotometer, and radioactivity was determined as described below.

Sedimentation coefficients ($S^{0}_{20,w}$) for C. acanthocephali RNAs were calculated (28) from the distribution of these RNAs in the gradient relative to ^{3}H-uridine-labeled (55,000 cpm/µg) wcRNA from Drosophila melanogaster embryonic culture cells (obtained from D. Steward), or to ^{3}H-uridine-labeled 28S and 18S RNA from D. melanogaster (obtained from P. Huck).

Annealing of Single Strands of kDNA and RNA. Single heavy (H) or light (L) strands of kDNA (10) were mixed with appropriate molar amounts of either wcRNA or mt-assoc RNA (determined by absorbance at 260 nm), and 2 M sodium phosphate (RNase free, pH 6.8) was added to give a final concentration of 0.5 M. These mixtures were then stoppered in sterile test tubes and incubated for 28 hr at 60°C. The mixtures were then cooled to room temperature and glass distilled, sterile water and 2 M Tris-HCl (pH 6.8) were added to give a final volume of 7 ml and a final Tris-HCl concentration of 0.1 M. Native kDNA associations, or kDNA H strands or L strands were self-annealed under the same conditions but without RNA present, as controls. C. acanthocephali wcRNA was also self-annealed, in the absence of DNA, as a control.

Cesium Sulfate Equilibrium Buoyant Density Gradient Centrifugation. To the 7 ml solutions containing coannealed mixtures of kDNA and RNA or self-annealed kDNA or RNA, solid cesium sulfate was added to give a final refractive index of 1.3750 (ρ = 1.510 gms/cm^3). These solutions were centrifuged at 43,000 rpm for 72 hr at 20°C using a Beckman type 65 rotor. After centrifugation 6-drop fractions were collected. Alternate fractions were analyzed for radioactivity while the refractive indices of every tenth fraction were determined in order to obtain a density profile of the gradient (29).

In vivo Radioactive Labeling of Nucleic Acids. C. acanthocephali were uniformly labeled with ^{32}P by growth in culture medium containing 10 mC/l of ^{32}P-orthophosphoric acid (New England Nuclear) for at least 10 generations. RNA was labeled with ^{3}H by growth for at least 6 generations in culture medium containing 1.5 mC/l of ^{3}H-uridine (New England Nuclear).

Determinations of Radioactivity in Nucleic Acid Samples. Fractions from sucrose gradients, and cesium sulfate gradients were diluted to 0.5 ml with SSC (150 mM sodium chloride, 15 mM sodium citrate), and 75 µg of alkaline denatured (by incubation for 16 hr at 37°C in 0.1 N sodium hydroxide) carrier Salmon sperm DNA (1 mg/ml) was added. Ice cold 20% trichloracetic acid (TCA) was added to a final concentration of 5%, and, after mixing, the solution was placed for 1 hr at 4°C. Precipitated nucleic acids were collected on glass fiber filters (934AH, Reeve Angel). Repeated rinsing of the tubes with concentrated hydrochloric acid (90 ml/l) and sodium pyrophosphate (45 gm/l) ensured maximum recovery of the nucleic acids. The filters were dried at 60°C and radioactivity was determined using 10 ml of BBOT (8 gms/l toluene, scintillation grade, Packard) as a fluor in the Packard 3320 liquid scintillation system.

Nuclease S1 Analysis. Analysis of RNA/DNA hybrids and of H or L strands of kDNA with nuclease S1 (from Aspergillus oryzae: Miles) was carried out using a modification of the technique of Leong et al (30). A control experiment was first performed to ascertain the specificity of the enzyme. A sample containing 0.5 µg double-stranded, ^{3}H-thymidine-labeled DNA (20,000 cpm/µg) from Escherichia coli and a second sample containing 0.5 µg E. coli single-stranded ^{3}H-thymidine-labeled DNA (produced by incubation of double-stranded DNA for 16 hr at 37°C in 0.3 N sodium hydroxide) were separately precipitated with ethanol and resuspended in 2 ml of S1 digestion buffer (0.3 M sodium chloride, 30 mM sodium acetate (pH 4.5) and 3 mM zinc chloride). To each sample, 10 µg of single-stranded Salmon sperm DNA and 150 units of S1 nuclease were added and the mixture was incubated for 2 hr at 50° C. Each mixture was cooled in an ice water bath and, after addition of 100 µg carrier Salmon sperm DNA, precipitated with 5% TCA. The precipitates were collected on filters and radioactivities determined. The data from this experiment indicated that 98.4% of the E. coli double-stranded DNA was resistant to S1, while only 1.9% of the single-stranded DNA was resistant.

Separate samples of H and L strands of kDNA were prepared for S1 analysis as follows. Approximately 2 µg (1,000 cpm ^{32}P) of ethanol-precipitated H strands or L strands were suspended in 40 µl of a buffer containing 0.6 M sodium chloride, 10 mM Tris (pH 7.1) and 3 mM EDTA and incubated at 65°C for either 10 sec or 24 hr. RNA/DNA hybrids were prepared for S1 analysis by mixing H or L strands of kDNA with 10 to 100-fold excess of C. acanthocephali wcRNA or mt-assoc RNA and the mixtures were incubated at 65°C in the same buffer used for incubation of separated H and L strands. Samples containing

separated H and L kDNA strands, and samples containing coannealed kDNA strands and RNA were then diluted into 2 ml of S1 digestion, buffer digestions and determinations of the fractions of DNAs resisting digestion were carried out as described above for E. coli DNA.

Staining with Bacteriophage T4 Gene 32 Protein (31) To nucleic acid samples in 10 mM sodium phosphate (pH 7.4) and 1 mM EDTA, bacteriophage T4 gene 32 protein (donated by K. Thomas and B. M. Olivera, and stored at 4.4 mg/ml in 30% glycerol, 50 mM sodium chloride, 2 mM Tris (pH 7.8) 5 mM EDTA and 1 mM dithiothreitol) was added to give approximately 80 μg/ml final concentration. The mixture was incubated for 5 min at 37°C followed by addition of 0.2% gluteraldehyde, and incubated for an additional 10 min at 37°C. This mixture was diluted 10 fold into a solution containing 10 mM tricine (pH 9.0), 1 mM EDTA, and 50 μg/ml ethidium bromide. Monolayers were then prepared on parlodion grids, stained with uranyl acetate, and rotary shadowed for electron microscope examination.

Electron Microscopy. Kintoplast DNA was prepared for electron microscopy by the formamide-protein monolayer technique of Davis et al. (32) using 50% and 20% formamide in the hyperphase and hypophase respectively. Other details of electron microscopy (including the use of bacteriophage fd single-stranded and double-stranded (RF) DNA as molecular weight standards) were as given in Fouts et al. (10,11).

RESULTS

Sucrose Gradient Analyses of RNAs. Sucrose gradient analysis of Crithidia acanthocephali whole cell RNA (wcRNA) revealed three major components (Fig. 1A) which, from the relative distribution of D. melanogaster wcRNA (28S, 18S, and 4S;(33)) had mean sedimentation coefficients of 25S, 18S and 4S (Fig. 1B and C). Similar values were found for wcRNA isolated by either the phenol/m-cresol, or the cesium chloride procedure. The majority of RNA extracted from a mitochondrion-enriched fraction of C. acanthocephali cells sedimented as a single band with a mean sedimentation coefficient of 17S (Fig. 2). A second band with a mean sedimentation coefficient of 3-4S was present in some gradients.

Cesium Sulfate Buoyant Density Gradient Analyses. Native kDNA associations, self-annealed kDNA H strands, and self-annealed kDNA L strands, when centrifuged to equilibrium separately in a cesium sulfate buoyant density gradient, each formed a single band with mean densities of 1.432 g/cm^3 (Fig. 3A), 1.465 g/cm^3 (Fig. 3B), and 1.434 g/cm^3 (Fig. 3C) respectively. The re-

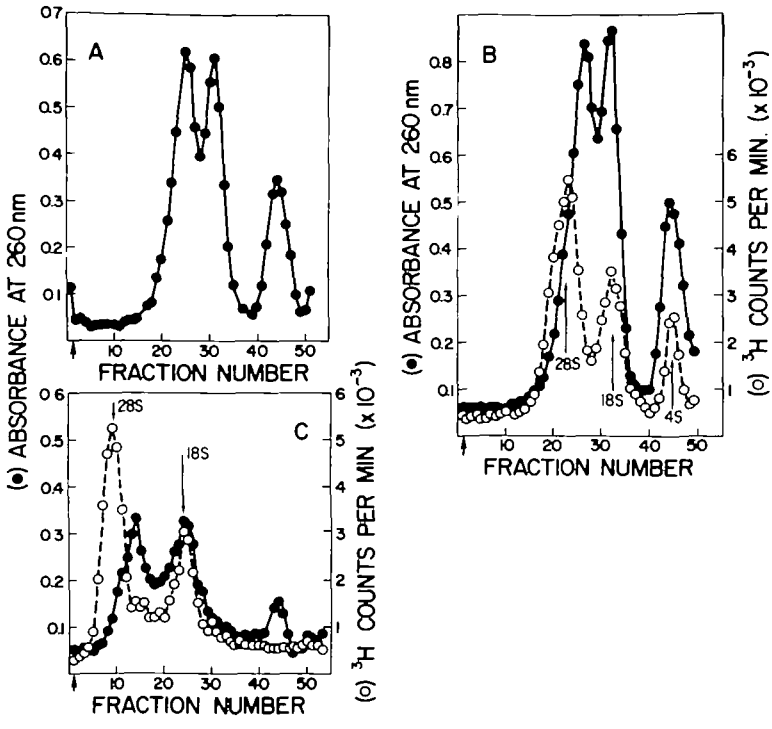

Figure 1. The distribution of C. acanthocephali wcRNA (solid circles, A_{260}) in 5%-20% sucrose gradients following centrifugation for 4.5 or 5 hours at 37,000 rpm in a Beckman SW41 rotor. The arrow below the abscissa in each frame indicates the bottom of the gradient. A. C. acanthocephali wcRNA isolated by the phenol/m-cresol procedure. B. As A except that D. melanogaster ^{3}H-labeled wcRNA (open circles) was added immediately before centrifugation. C. C. acanthocephali wcRNA isolated by the cesium chloride procedure, to which D. melanogaster ^{3}H-labeled 28S and 18S RNA (open circles) was added immediately before centrifugation.

lationships of these buoyant density values were confirmed from the results of cocentrifugation of native associations with self-annealed H strands (Fig. 3D) and self-annealed L strands (Fig. 3E). At equilibrium in a cesium sulphate buoyant density gradient wcRNA was also in a single band, with a mean density of 1.660 g/cm^3 (Fig. 3F).

^{32}P-labeled kDNA H strands or L strands was coannealed with unlabeled wcRNA in ratios of from 1:1 to 1:100, and the products centrifuged to equilibrium in cesium sulphate gradients. The pertinent results for experiments involving kDNA H strands are shown in Fig. 4. For the sample containing

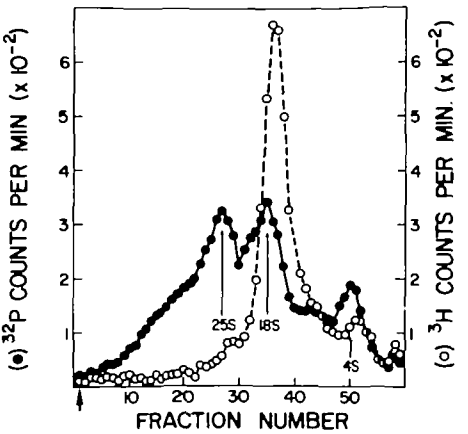

Figure 2. The relative distribution of C. acanthocephali ^{32}P-labeled wcRNA (solid circles) and of ^{3}H-labeled mt-assoc RNA (open circles) in a 5%-20% sucrose gradient following centrifugation for 5 hrs at 37,000 rpm in a Beckman SW41 rotor. The arrow under the abscissa indicates the bottom of the gradient. The sedimentation coefficients (25S, 18S, and 4S) shown are for wcRNA calculated from the data shown in Fig. 1.

equal weights of DNA and RNA, the DNA-associated radioactivity formed a single band with a mean density characteristic of the kDNA H strands. However, at a ratio of DNA:RNA of 1:4, the DNA-associated radioactivity formed a broad band with two distinct peaks with mean buoyant densities of 1.498 and 1.466 g/cm^3. The proportion of radioactivity under the denser peak increased with increase in proportion of RNA in the coannealing mixture. At a DNA:RNA ratio of 1:10 all of the radioactivity appeared as a single narrow band with a mean buoyant density of 1.497 g/cm^3 (Fig. 4D). A similar result was found for DNA:RNA ratios up to 1:100. Hybrid formation between kDNA H strands and a component (or components) of wcRNA could account for the increase in buoyant density of kDNA H strands (34). The observation that the buoyant density increase was uniform for all detectable kDNA H strands when annealing involved a 10 fold or more excess of wcRNA suggests that a saturable fraction of each H strand is being hybridized by RNA.

In contrast, the experiments with kDNA L strands did not provide evidence for a wcRNA component complementary to these strands. For coannealed mixtures of kDNA L strands and wcRNA up to a ratio of 1:100, the DNA associated radioactivity formed a single band at a mean density (1.435 g/cm^3) characteristic of kDNA L strands (Fig. 5B).

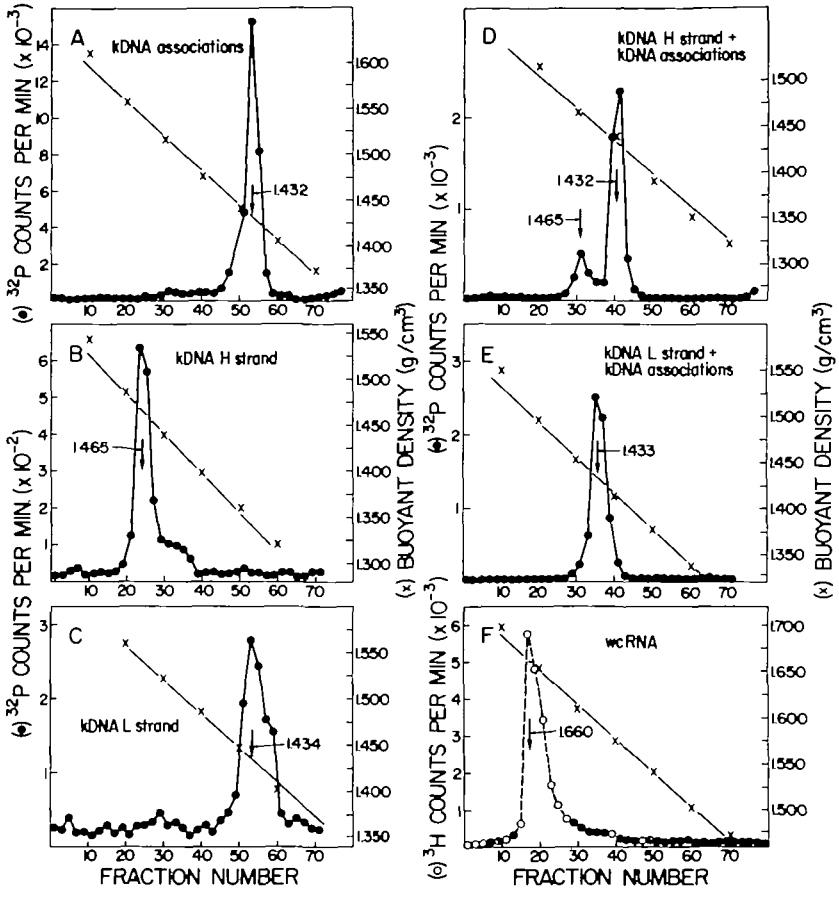

Figure 3. The distribution of ^{32}P-labeled C. acanthocephali kDNA associations, self-annealed H strands and self-annealed L strands (A-E, solid circles) singly and in the combinations indicated, and of ^{3}H-labeled C. acanthocephali wcRNA (F) following centrifugation to equilibrium in cesium sulphate buoyant density gradients. Buoyant densities indicated by arrows in the figures were determined from the density profile (X-X) of each gradient.

In order to test the hypothesis that the observed shift to a higher buoyant density of kDNA H strands following its coannealing with wcRNA represented RNA/DNA hybrid formation, two further experiments were conducted.

RNA can be hydrolyzed and removed from an RNA/DNA hybrid by alkali treatment (34, 35). A portion of the product of coannealing ^{32}P-labeled kDNA H strands and wcRNA was therefore treated with 0.1 N NaOH at 37°C for

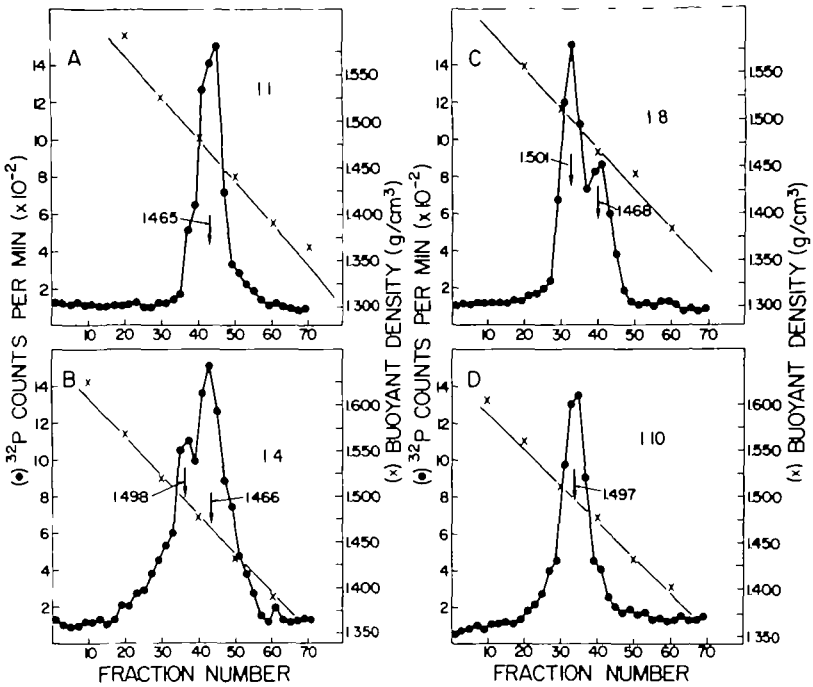

Figure 4. The distribution of C. acanthocephali ^{32}P-labeled kDNA H strands (solid circles) coannealed with unlabeled wcRNA in the proportions shown in the upper right of each frame (A-D), following centrifugation to equilibrium in cesium sulphate buoyant density gradients. Buoyant densities indicated by arrows in the figures were determined from the density profile (X-X) of each gradient.

8 hr. The mixture was then adjusted to pH 6.8 with HCl, and incubated at 60° C for 18 hr. Following centrifugation to equilibrium in a cesium sulphate buoyant density gradient, the DNA-associated radioactivity was distributed in a single band which had a mean buoyant density characteristic of unhybridized kDNA H strands (ρ = 1.465 g/cm^3, Fig. 5C).

RNA which is hybridized to DNA, unlike unhybridized single-strand RNA is resistant to digestion by ribonuclease A (RNase A) and Ribonuclease T1 (RNase T1) when the reaction is attempted in 2 X SSC (36-38). A portion of the product of coannealing ^{32}P-labeled kDNA H strands and wcRNA was dialyzed into 2 X SSC, and approximately 10,000 cpm (0.4 µg) of ^{14}C-labeled Chinese hamster ovary cell RNA (obtained from D. Kolokovsky; shown to comprise 28S, 18S and 4S components) was added as a control, and the mixture was incubated

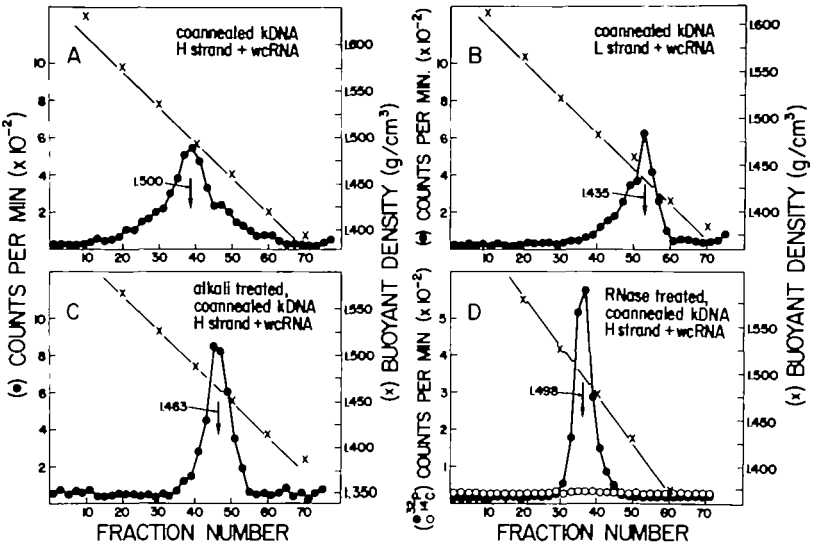

Figure 5. The distributions at equilibrium in cesium sulphate gradients of C. acanthocephali ^{32}P-labeled kDNA H strands and L strands (solid circles), coannealed with a 20-fold excess of wcRNA (A and B), and followed by alkali treatment (C) or RNase digestion (D)). The profiles shown in D are from a gradient that contained a sample of kDNA H strands (solid circles) which had been coannealed with wcRNA, and a sample of ^{14}C-labeled Chinese hamster ovary RNA (open circles), the two samples having been mixed and then incubated for 1 hr at 37°C in 2 X SSC containing 50 µg/ml RNase A and 5 units/ml RNase T1. Buoyant densities indicated by arrows in the figures were determined from the density profile (X-X) of each gradient.

for 1 hr at 37°C with 50 µg/ml RNase A (freed from DNase activity by heating at 80°C for 5 min in 0.1 M sodium acetate (pH 5.0) before use), and 5 units/ml RNase T1 (pretreated as for RNase A). Following centrifugation to equilibrium in a cesium sulphate buoyant density gradient, the ^{14}C-radioactivity was evenly distributed throughout the gradient (Fig. 5 D) indicating that the Chinese hamster cell single-stranded RNA had been degraded by the enzyme digestion. In contrast, kDNA-associated radioactivity formed a single band with a mean buoyant density of 1.498 g/cm^3 similar to that found for the undigested product of coannealing kDNA H strands and wcRNA. These two results are clearly consistent with the interpretation that a component of wcRNA is complementary to each of the kDNA H strands.

In view of these results, it seemed reasonable to ask next whether species of RNA homologous to small circular kDNA were to be found associated

with mitochondria, and if so, at what relative concentrations. Therefore, mt-assoc RNA was annealed with ^{32}P-labeled kDNA H strands or kDNA L strands, at DNA:RNA ratios of 1:5 to 1:20 and the products analyzed by cesium sulphate buoyant density gradient centrifugation (data not shown). Annealing of this RNA fraction with kDNA H strands resulted in a shift in buoyant density of the DNA-associated radioactivity from 1.465 g/cm^3 to 1.499 g/cm^3, similar to that found following annealing of wcRNA with kDNA H strands. All DNA in the samples was found at the higher buoyant density (1.499 g/cm^3) only when the ratio of mt-assoc RNA:DNA was 8:1 or greater. A shift in buoyant density of the DNA-associated radioactivity was not observed upon analysis of the co-annealed product of mt-assoc RNA and L strand kDNA. These data suggest that a component(s) of the mt-assoc RNA fraction is complementary to kDNA H strands, and is the same RNA as that detected in wcRNA.

The contention that wcRNA and mt-assoc RNA of C. acanthocephali contain a component complementary to each kDNA H strand but not to kDNA L strands was strengthened by the results of the following experiment. ^{32}P-labeled kDNA H strands and L strands were annealed separately to an 8-fold excess of either ^{3}H-uridine-labeled wcRNA or ^{3}H-uridine-labeled mt-assoc RNA and the products were analyzed by cesium sulphate equilibrium buoyant density centrifugation. The results are shown in Fig. 6. As was found previously for an 8:1 mixture of wcRNA:kDNA H strands (Fig. 4C), the majority of the DNA-associated ^{32}P-radioactivity banded with a mean buoyant density of 1.501 g/cm^3, while a minority banded with a mean buoyant density (1.465 g/cm^3) characteristic of self-annealed kDNA H strands (Fig. 6A). While most of the RNA-associated ^{3}H-radioactivity was located at a much higher density than the kDNA, a minor fraction with a peak buoyant density of 1.515 g/cm^3 overlapped the denser kDNA fraction, consistent with RNA/DNA hybrid formation. A similar result was obtained for the coannealed product of kDNA H strands and mt-assoc RNA (Fig. 6B). The observation that in each experiment, the minor peak of RNA-associated ^{3}H-radioactivity was at a higher buoyant density than the corresponding peak of DNA-associated ^{32}P-radioactivity, suggests that some of the DNA molecules present in each gradient were of less than unit-length.

In contrast to the results obtained using kDNA H-strands, cesium sulphate buoyant density gradient analysis of the products of annealing kDNA L strands to either wcRNA or mt-assoc RNA failed to provide evidence of RNA/DNA hybrid formation. In both cases (Fig. 6C and D) the DNA-associated radioactivity appeared as a single band with a buoyant density characteristic of self-annealed kDNA L strands and none of the RNA-associated ^{3}H radioactivity ap-

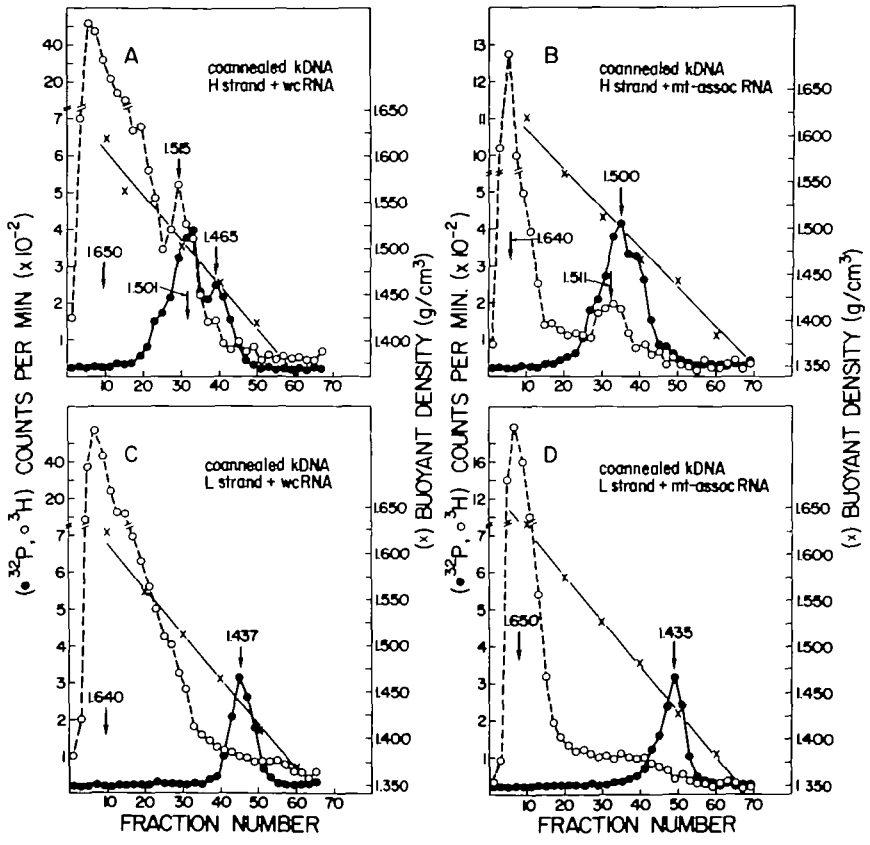

Figure 6. The distribution of C. acanthocephali ^{32}P-labeled kDNA H strands and L strands (solid circles) and ^{3}H-labeled wcRNA and mt-assoc RNA (open circles) in cesium sulphate buoyant density gradients following centrifugation to equilibrium of the products of coannealing the different DNAs and RNAs. Buoyant densities indicated by arrows in the figures were determined from the density profile (X-X) of each gradient.

peared to be at densities coincident with the L strand DNA.

S1 Nuclease Analysis. In order to determine the proportion of each kDNA H strand which forms a hybrid with a component of wcRNA or mt-assoc RNA, we determined the resistance of DNA in DNA/RNA hybrids to the nuclease S1 from Aspergillus oryzae. This nuclease degrades single-stranded DNA but not double-stranded DNA, or DNA duplexed with RNA (30, 39). The results are summarized in Table 1. When self-annealed kDNA H strands and self-annealed kDNA L strands were separately digested with S1, 11.4% and 9.2% respectively

Table 1. Results of S1 nuclease digestion of separated kDNA H and L strands following self-annealing, and coannealing with wcRNA and mt-assoc RNA.

Substrate	Number of digestions	% (±SD) DNA resistant to S1 digestion[a]
Self-annealed kDNA H strands	4[b]	11.4 ± 1.1
Self-annealed kDNA L strands	2[c]	9.2 ± 0.7
Coannealed kDNA H strands + wcRNA	6[d]	21.6 ± 2.0
Coannealed kDNA H stranas + mt-assoc RNA	8[e]	21.4 ± 1.7
Coannealed kDNA L strands + wcRNA	4[f]	10.4 ± 2.1

a. Standardized against the resistance to S1 digestion of E. coli double-stranded DNA (see Material and Methods).

b. Two digestions, each, of kDNA H strands self-annealed for 10 sec and 24 hr.

c. One digestion, each, of kDNA L strands self-annealed for 10 sec and 24 hr.

d. Two digestions, each, of the products of coannealing kDNA and wcRNA in the ratios 1:10, 1:40, and 1:60.

e. Two digestions, each, of the products of coannealing kDNA and mt-assoc RNA in the ratios 1:10, 1:40, 1:60, and 1:80.

f. Two digestions, each, of the products of coannealing kDNA and wcRNA in the ratios 1:10 and 1:100.

were found to be resistant to degradation. The finding that the time of annealing did not influence the proportion of H strand or L strand kDNA resistant to S1 digestion (Table 1, footnotes b and c) suggests that the individual strands contain duplexed regions resulting from annealing of inverted repeat sequences (40). Following digestion with nuclease S1 of the products of coannealing kDNA H strands with wcRNA, and kDNA H strands with mt-assoc RNA, 21.6% and 21.4% respectively of the kDNA remained undegraded. In contrast, only 10.4% of the kDNA L strands remained undegraded following digestion of the coannealed product of this DNA and wcRNA with nuclease S1. These results clearly indicate that RNA molecule(s) present in both wcRNA and mt-assoc RNA are complementary to a total of approximately 10% of each kDNA H strand.

Gene 32 Protein Staining. In order to determine whether the complementary RNA hybridizes to a single continuous region of the kDNA H strand or to multiple separated smaller regions of the H strand totalling 10% of its length, we stained wcRNA/kDNA H strand hybrids with gene 32 protein from

bacteriophage T4, and examined the products in the electron microscope. Gene 32 protein binds preferentially to single-stranded regions of nucleic acids (41) and these regions appear thicker than double-stranded regions when prepared for electron microscopy under specific conditions (31).

First, as controls, self-annealed kDNA H strands, and a mixture of kDNA H strands and covalently-closed double-stranded circular molecules of kDNA were prepared for electron microscopy following staining with gene 32 protein. The entire length of all the circular molecules in the preparation of self-annealed H strand kDNA was of a similar, uniform thickness. In the preparation containing a mixture of single-stranded and double-stranded molecules, the entire length of some of the circular molecules was of the same uniform thickness as was observed for circular molecules in the preparation containing only kDNA H strands (Fig. 7A). The remainder of the circular molecules were strikingly thinner, again along their entire lengths (Fig. 7A). These observations make clear that we can distinguish single-stranded and

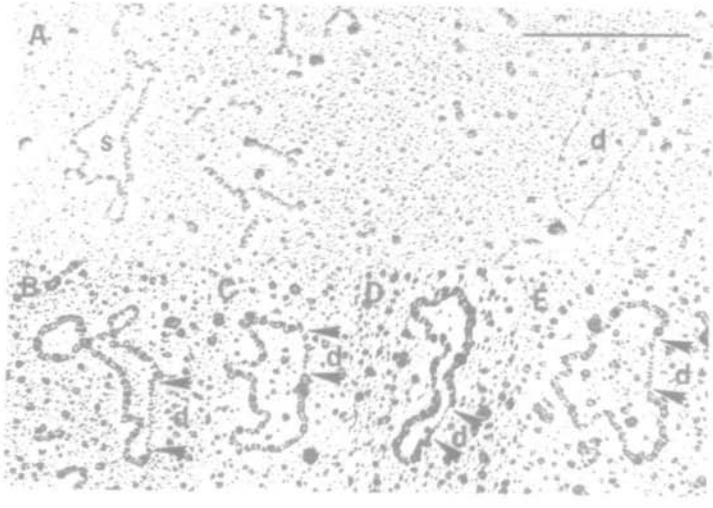

Figure 7. Electron micrographs of rotary shadowed molecules of C. acanthocephali kDNA stained with gene 32 protein from bacteriophage T4. A. From a mixture of H strands and covalently-closed, single circular, double-stranded molecules. The double-stranded, covalently-closed circular molecule (d) has a distinctly thinner appearance than the circular single-stranded H strand molecule(s). B-E. Single circular H-strand molecules taken at equilibrium from the buoyant density region 1.500 - 1.510 g/cm^3 of a cesium sulphate gradient of kDNA H strand which had been annealed with wcRNA. Each molecule contains a single region (d) averaging 11.4% of the circular contour length, which is distinctly thinner than the rest of the molecule, indicating that it is double stranded. The bar in A (upper right) equals 0.5 μ.

double-stranded kDNA in electron microscope preparations following gene 32 protein staining. It was determined that in the second preparation described, the ratio of the contour length of circular single-stranded molecules (n=30) to the contour length of circular double-stranded molecules (n=30) was 0.995.

Next, kDNA H strands and wcRNA were coannealed in a ratio of 1:10 and the product was centrifuged to equilibrium in a cesium sulphate buoyant density gradient. The gradient was fractionated and the fractions containing the buoyant density region 1.500 to 1.510 g/cm^3 (expected to include presumptive RNA/DNA hybrids) were pooled and prepared for electron microscopy following staining with gene 32 protein. Examination of the preparations revealed 1.6 daltons circular and linear molecules, and shorter linear molecules. A single region with a mean length equal to 11.4 $\pm$ 0.15% (n=30) of the contour length of each of 91.5% of the circular molecules had a distinctly thinner appearance (Fig. 7B-E) than the remainder of the molecule, indicating that this region was a duplex structure. The entire contour length of each of the remaining 8.5% of the circular molecules was of the uniform thickness expected for a totally single-stranded molecule. The majority of unit-length linear molecules in this preparation also contained a single, thin region accounting for approximately 10% of the total molecule length.

These data suggest that a component (or components) of wcRNA is complementary to a single region of each kDNA H strand representing approximately 10% of its length.

DISCUSSION

The data presented indicate that a component (or components) of RNA isolated from whole cells, or from a mitochondrion-enriched fraction, of C. acanthocephali is complementary to a single section equal to approximately 10% of the H strand of the small circular (1.58 x 10^6 dalton) molecules of the kDNA of this organism. It seems reasonable to conclude therefore that at least part of the small circular kDNA molecule is transcribed. It should be noted, however, that at least in C. acanthocephali it is not ruled out that DNA sequences complementary to the RNA molecule of interest are present in nuclear DNA. If the complementary RNA is a single molecule it would be approximately 240 nucleotides long, and could code for a protein comprising about 80 amino acids.

Whether or not the complementary RNA molecule we have detected represents the total extent of transcription of small circular kDNA is not established by our data. If RNA transcripts of different stability were produced,

such as has been reported from mammalian mitochondrial DNA (42, 43) then our experiments would only have detected the more stable forms.

From a consideration of the ratio of wcRNA to H strand kDNA necessary to involve all kDNA H strands in hybridization, and of the proportion of each H strand hybridized, it appears that the RNA complementary to kDNA accounts for approximately 1% of wcRNA. The sedimentation coefficients (25S and 18S) of the two major fractions of wcRNA were similar to the values reported for the ribosomal RNAs of C. oncopelti (4), C. fasciculata (22), Leishmania donovani (38), and L. tarentolae (23). Mitochondrion-assoc RNA contained approximately the same proportion (1%) of RNA complementary to kDNA H strands as did wcRNA. This may indicate that, following transcription in the mitochondrion, kDNA complementary-RNA is transported to the cytoplasm. It seems likely, by comparison with the results of others (22, 4), that the major component of mt-assoc RNA (17S) is a cytoplasmic RNA contaminant.

The data from the S1 digestion experiments reported here which indicate that duplex regions accounting for about 10% of the molecule length readily form in separated H strands and L strands, are in agreement with the results of our previously reported studies of the kinetics of renaturation of this DNA (10), and are consistent with the presence in kDNA of inverted repeat sequences (40). As duplex regions were not observed in unhybridized, separated L or H strands of kDNA prepared for electron microscopy, either in the presence of formamide or following gene-32 protein staining, the S1-digestion results might best be accounted for by the occurrence in kDNA of small inverted repeat sequences scattered through the molecule.

Four sedimentation species of RNA, 14.6S, 11.4S, 10.1S, and 6.9S, the syntheses of which were inhibited by ethidium bromide were identified by Nichols and Cross (22) from mitochondrial fractions of C. fasciculata. However, no further attempt was made to determine the identity of the DNA molecules to which these RNAs were complementary. Simpson and Simpson (23) isolated two major RNA species with sedimentation coefficients of 9S and 12S from mitochondrial fractions of L. tarentolae and showed that both of these RNA molecules hybridize selectively to the large circular component of the kDNA of this organism. They argued that this observation and the sizes and base compositions of the 9S and 12S RNA molecules made it improbable that these RNAs were in fact coded by the small circular kDNA component. From the results of hybridization experiments involving restriction fragments of kDNA and wcRNA of C. luciliae, Hoeijmakers and Borst (24) detected RNA totaling 2300 to 2500 nucleotides complementary to the large circular com-

ponent (22 x 10^6 daltons) of the kDNA. RNA complementary to the small circular component of the kDNA (1.5 x 10^6 daltons) was not found, leading these workers to conclude that the small circular molecules do not have a coding function.

As the conclusion of Hoeijmakers and Borst (24) for C. luciliae kDNA is contrary to the conclusion we draw from the present study, that the small circular component of C. acanthocephali kDNA is partially transcribed, the following considerations are important. The kDNA used in all of our experiments was selected and characterized as the small circular component at all stages during isolation (for details see Reference 10). This included the determination of the highly characteristic alkaline and neutral buoyant densities of the separated kDNA strands, and the visualization of 1.58 x 10^6 daltons circular and linear molecules in electron microscope preparations. There is evidence from our previous renaturation and restriction enzyme studies (10, 11) that kDNA of C. acanthocephali includes a molecule with a higher sequence complexity than the 1.58 x 10^6 daltons circular component. However, the possibility that minor contamination by the component of higher sequence complexity could account for the observed shift in buoyant density following RNA/kDNA hybridization in the present experiments is ruled out, as all detectable H strand kDNA in the preparations was involved in the density shifts. Furthermore, in electron microscope preparations of H strand kDNA which had been annealed with wcRNA we observed 1.58 x 10^6 dalton circular molecules containing a duplex region of a size predictable for an RNA/kDNA hybrid from our other experiments.

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REFERENCES

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1. Honigberg, B. M., Balamuth, W., Bovee, E. C., Corliss, J. O., Gojidics, M., Hall, R. P., Kudo, R. R., Levine, N. D., Loeblich, A. R., Weiser, J. and Wenrich, D. H. (1964) J. Protozool. 11, 7-20.
2. Renger, H. C. and Wolstenholme, D. R. (1972) J. Cell Biol. 54, 346-364.
3. Riou, G. and Delain, E. (1968) Proc. Natl. Acad. Sci. U.S.A. 62, 210-217.
4. Laurent, M. and Steinert, M. (1970) Proc. Natl. Acad. Sci. 66, 419-424.
5. Renger, H. C. and Wolstenholme, D. R. (1970) J. Cell Biol. 47, 689-702.
6. Renger, H. C. and Wolstenholme, D. R. (1971) J. Cell Biol. 50, 533-540.
7. Wolstenholme, D. R., Renger, H. C., Manning, J. E. and Fouts, D. L. (1974) J. Protozool. 21, 622-631.
8. Simpson, L. and DaSilva, A. (1971) J. Mol. Biol. 56, 443-473.
9. Cheng, D. and Simpson. L. (1978) Plasmid 1, 297.
10. Fouts, D. L., Manning, J. E. and Wolstenholme, D. R. (1975) J. Cell Biol. 67, 378-399.
11. Fouts, D. L., Wolstenholme, D. R. and Boyer, H. W. (1978) J. Cell Biol. 79, 329-341.
12. Kleisen, C. M. and Borst, P. (1975) Biochim. Biophys. Acta 407, 473-478.
13. Kleisen, C. M., Borst, P. and Weijers. (1976a) Eur. J. Biochem. 64, 141-151.
14. Kleisen, C. M., Weislogel, P. O., Fonck, K. and Borst, P. (1976b). Eur. J. Biochem. 64, 153-160.
15. Weislogel, P. O., Hoeijmakers, J. H. J., Fairlamb, A. H., Kleisen, C. M. and Borst, P. (1977) Biochim. Biophys. Acta 478, 167-179.
16. Borst, P., Fase-Fowler, F., Steinert, M. and van Assel, S. (1977) Exp. Cell Res. 110, 167-173.
17. Fairlamb, A. H., Weislogel, P. O., Hoeijmakers, J. H. J. and Borst, P. (1978) J. Cell Biol. 76, 293-309.
18. Wesley, R. D. and Simpson, L. 1973a. Biochim. Biophys. Acta 319, 254-266.
19. Wesley, R. D. and Simpson, L. 1973b. Biochim. Biophys. Acta 319, 267-280.
20. Simpson, L. and Hyman, B. (1970) in Biochemistry of Parasites and Host-Parasite Relationships, ed., Van den Bossche, H. (Amsterdam, North Holland) pp. 219.
21. Steinert, M. and van Assel, S. (1976) Exp. Cell Res. 96, 406-409.
22. Nichols, J. M. and Cross, G. A. M. (1977) J. Gen. Microbiol. 99, 291-300.
23. Simpson, L. and Simpson, A. (1978) Cell 14, 169-178.
24. Hoeijmakers,J. H. J. and Borst, P. (1978) Biochim. Biophys. Acta 521, 407-411.
25. Watts, R. L. and Mathias, A. P. (1967) Biochim. Biophys. Acta 145, 828-831.
26. Kirby, K. S. (1968) in Methods in Enzymology, eds., Grossman L. and Moldave, K. (Academic Press, New York) Vol 12B, 87-99.
27. Glisin, V., Cekvenjakov, C. and Byus, C. (1974) Biochemistry 13, 2633-2637.
28. McConkey, E. H. (1967) in Methods in Enzymology, eds., Grossman, L. and Moldave, K. (Academic Press, New York) Vol. 12A, 620-634.
29. Vinograd, J., and Hearst, J. E. (1962) Fortshr. Chem. Org. Naturstoffe 20, 372-422.

30. Leong, J-A., Garapin, A-C, Jackson, N., Levinson, W. and Bishop, J. M. (1972) J. Virol. 9, 891-902.
31. Wu, M. and Davidson, N. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 4506-4510.
32. Davis, R. W., Simon, M. and Davidson, N. (1971) in Methods in Enzymology, eds., Grossman L. and Moldave, K. (Academic Press, New York) Vol. 21, 413-428.
33. Rittosa, F. M., Atwood, K. C. and Spiegelman, S. (1966) Genetics 54, 663-676.
34. Szybalski, W., Kubinski, H., Hradecna, Z. and Summers, W. C. (1971) in Methods in Enzymology, eds., Grossman, L. and Moldave, K. (Academic Press, New York) Vol. 21, 383-413.
35. Davidson, J. N. (1969) The Biochemistry of Nucleic Acids (Methuen, London) 6th edn., pp. 240.
36. Thomas, J. R. and Tewari, K. K. (1974) Biochim. Biophys. Acta 361, 73-83.
37. Quintrell, N., Varmus, H. E., Bishop, J. M., Nicholson, M. O. and McAllister, R. M. (1974) Virology 58, 568-575.
38. Leon, W., Fouts, D. L. and Manning, J. (1978) Nucleic Acids Research 5, 491-504.
39. Sutton, W. D. (1971) Biochim. Biophys. Acta 240, 522-531.
40. Wilson, D. A. and Thomas, C. A. Jr. (1974) J. Mol. Biol. 84, 115-144.
41. Delius, H., Mantell, N. J. and Alberts, B. (1972) J. Mol. Biol. 67, 341-350.
42. Aloni, Y., and Attardi, G. (1971) J. Mol. Biol. 55, 251-267.
43. Murphy, W. I., Attardi, B., Tu, C., and Attardi, G. (1975) J. Mol. Biol. 99, 809-814.
44. Spencer, R., and Cross, G. A. M. (1976) J. Gen. Microbiol. 93, 82-88.