Different base per unit length ratios exist in single-stranded RNA and single-stranded DNA

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ABSTRACT

A significant difference was found to exist in the number of bases per unit length of single-stranded RNA as compared to single-stranded DNA when single-stranded RNA or DNA molecules of known nucleotide sequence were measured by electron microscopy using a cytochrome spreading technique.

Using this technique, single-stranded RNA was found to have 17.5% more bases per unit of length than single-stranded DNA. These ratios were verified using four different denaturing conditions for the RNA: 80% formamide, 80% formamide plus glyoxal, 80% formamide/4M urea and 80% formamide/4M urea plus glyoxal.

Molecules ranging in size from 1541 to 5386 nucleotides were examined and the number of bases per unit length was found to vary inversely with the absolute length of the molecules. The difference in ratio of bases per micrometer was consistent when RNA and DNA molecules of the same length were compared.

INTRODUCTION

Accurate determinations of the size of nucleic acids by electron microscopy are most commonly based on internal standards of known size. Using the basic protein film technique for visualization of nucleic acids with the electron microscope(1) DNA and RNA can be visualized by virtue of a sheath of denatured protein (usually cytochrome c) bound to the nucleic acid. Double-stranded DNAs of known sequence have been well characterized by this technique in terms of length and helical structure. Single-stranded nucleic acids however, have not been quantitated with comparable thoroughness. Additionally, the problem of accurate molecular weight determinations of single-stranded RNAs as compared with DNAs has been especially difficult for several reasons: 1) RNAs vary widely in the extent of their secondary structure, 2) It is difficult to achieve conditions that completely denature RNA molecules and 3) RNA standards of known sequence have not been available.

Recently the complete nucleotide sequence of four RNA molecules has

been determined; the RNAs of bacteriophages Q β (2), and MS2 (3) and the 16S (4) and 23S (5) ribosomal (r) RNAs of E. coli have been sequenced.

We needed to identify and characterize suitable internal standards for determination of the molecular size of single-stranded RNAs of unknown length. To do so we measured the contours of the four sequenced RNA molecules by electron microscopy and determined their base per unit length ratios under four different denaturing conditions. For comparison we determined the contour length of the following DNAs: 1) single-strand circular ϕ X174 virion DNA which has 5386 nucleotides (6), 2) the single-strand linear full length DNA of ϕ X174 and 3) two single-strand ϕ X174 DNA fragments of 2748 and 1697 bases, respectively, that were generated by restriction endonuclease digestion of ϕ X174 RF and subsequent denaturation.

The results of these studies, reported here, reveal a significant difference in the base per unit length ratios of DNA and RNA as measured by electron microscopy. These results show not only that single-stranded PNA has an average of 17.5% more bases per unit length than single-stranded DNA but also that for both RNA and for DNA there is an inverse relationship between molecular length and bases per unit length.

MATERIALS AND METHODS

Reagents. Cytochrome c (equine heart, salt free, grade A) was purchased from Calbiochem. Two grades of formamide were employed. Hyperphase formamide was from Matheson, Coleman, and Bell. Hypophase formamide (reagent grade) was from Fisher. Glyoxal (technical, 40% in water) was obtained from Eastman. Urea (ultra pure) was purchased from Becton Dickenson.

Restriction endonucleases Xho I and Hpa II were from New England Biolabs and Boehringer Mannheim respectively.

Purified E. coli 16S and 23S ribosomal, MS2 and Q β RNAs were purchased from Miles Laboratories.

ϕ X174 phage DNA and ϕ X174 replicative form (RF) DNA were gifts from Sandra Phillips.

RNA Preparation. Purified Q β and MS2 RNAs were obtained in lyophilized form, and dissolved in TE buffer (0.01 M Tris, pH 7.4, 0.001 M EDTA) immediately before use. The 16S and 23S E. coli ribosomal RNAs were separated by two consecutive cycles of velocity centrifugation, collected by ethanol precipitation, and dissolved in TE buffer immediately prior to use.

The four RNA species were monitored for purity and homogeneity by electrophoresis on denaturing agarose-urea gels (pH 3.0) (7) and the RNA

was visualized by staining with methylene blue. Each RNA migrated as one distinct species; no degradation was discernable.

DNA Preparation. Single stranded linear DNA molecules were prepared by digesting duplex ϕ X174 RF DNA with the desired restriction endonuclease. The products were denatured with glyoxal to yield single-stranded molecules. Linear full length DNA was the product of digestion with Xho I. (8) ϕ X174 DNA fragments of 2748 and 1697 base pairs were generated using the restriction endonuclease Hpa II (9,10). Hpa II produces five restriction products, two of which, the 2748 and 1697 base pair fragments, were of interest in this study. The Hpa II fragments were separated on a 1% agarose gel and visualized by staining with ethidium bromide. The separated fragments were recovered from the gel by electroelution, concentrated by ethanol precipitation and dissolved in TE buffer before use.

Glyoxal Denaturation. RNA and DNA were denatured using glyoxal according to the following procedures. Nucleic acid solutions in TE buffer were incubated for one hour at 37° in a mixture of 0.5M glyoxal, 60% formamide, 0.01 M NaH_2PO_4 - Na_2HPO_4 buffer pH 6.9, and 0.001 M EDTA. This solution was diluted ten-fold with hyperphase reagents before spreading.

Electron Microscopy. RNA molecules were spread for electron microscopy using four different denaturing conditions: (1) 80% formamide spreading, (2) urea-formamide (4M urea-80% formamide) spreading, (3) 80% formamide spreading with a glyoxal pre-treatment and (4) urea-formamide (4M urea-80% formamide) spreading with a glyoxal pre-treatment. Restriction endonuclease cut DNA was spread using 80% formamide with a glyoxal pretreatment to denature the double strands.

Nucleic acid samples were diluted such that the final hyperphase solutions were 80% formamide or formamide-urea, 0.1 M Tris, pH 8.5, 0.01 M EDTA, 30-60 $\mu\text{g/ml}$ cytochrome c, 0.25-1.0 $\mu\text{g/ml}$ RNA or linear DNA and 0.1 $\mu\text{g/ml}$ ϕ X174 single stranded DNA. The ϕ X174 phage DNA was used as an internal standard in every spread. For the glyoxal pretreatment spreads, ϕ X174 single-stranded DNA was added to the incubation mixture. Because ϕ X174 phage DNA is a covalently closed circle it was easily discernable from the linear RNA and DNA.

Additionally, all measurements were confirmed using PIPES, a sulfonic acid buffer at pH 6.5, which does not react with glyoxal, instead of the Tris buffer. Essentially identical results were obtained with the two buffer systems.

100 μl of hyperphase solution was layered on a glass slide ramp so that

it flowed onto a hypophase solution whose available surface area was approximately 40 cm².

Hypophase solutions for 80% formamide spreads contained 50% formamide (reagent grade), 50% distilled water containing 0.01 M Tris, pH 8.5, and 0.001 M EDTA. The urea-formamide technique employs a hypophase of 30% formamide, 70% distilled water, 0.01M Tris pH 8.5 and 0.001M EDTA. The sample was picked up on a parlodion coated copper grid. The grids were stained in 80% ethanol containing uranyl acetate, air dried, and rotary-shadowed at a 10° angle with platinum-palladium (80:20). For increased parlodion stability, a thin carbon coating was sublimated onto the grids.

Micrographs were taken at 7000-9000 x magnification using a Hitachi H500 electron microscope. The magnification was repeatedly calibrated with a grating replica having a spacing of 2160 lines per mm. Length measurements were made by projecting electron micrograph negatives onto a Hewlett-Packard 9864A digitizer coupled to a Hewlett-Packard 9825 calculator, and tracing the molecular contour with the digitizer's cursor. The calculator was programmed with appropriate jitter and smoothing factors. Only molecules which were non-overlapping were measured.

RESULTS

Measurement of single-strand RNA and DNA molecules by electron microscopy. Recently, the complete sequence of four RNA molecules, the genomic RNAs of the bacteriophages Q β and MS2 and the *E. coli* ribosomal 16S and 23S RNAs have been determined (2,3,4,5). Samples of each of these RNAs were prepared for electron microscopy as described in Methods. Four different denaturing conditions were employed: 80% formamide, 80% formamide plus glyoxal, urea-formamide and urea-formamide plus glyoxal.

In each preparation, circular, single-stranded ϕ X174 DNA molecules, easily distinguished from the linear RNAs, were included as an internal standard. Electron micrographs of the spread molecules were made from several locations on each grid. A minimum of two separate grids was used for each RNA species under each denaturing condition. One hundred RNA molecules and fifty ϕ X174 DNA molecules were measured from micrographs of each grid.

A representative electron micrograph of each RNA species examined, including a ϕ X174 circular molecule for reference, is shown in Figure 1.

The distributions of the molecular length measurements for each combination of the four RNAs under each of the four denaturing conditions are

Figure 1 Electron micrograph of *E. coli* 16S rRNA (a) and 23S rRNA (b), Q8 RNA (c), MS2 RNA (d). RNA was spread using the formamide plus glyoxal technique as described in methods. A SS circular ϕ X174 DNA molecule is included for reference in each field.

displayed as histograms in Figure 2.

From these data the molecular lengths of each of the four RNA molecules and the ϕ X174 DNA were determined. The average molecular length of each molecule and the standard deviations as determined from measuring 200 molecules for each denaturing condition is shown in Table 1.

This data shows that there is a 3.9% difference between length measurements made under the least denaturing condition (80% formamide) and the most denaturing condition (urea-formamide plus glyoxal). There is less than a 1.5% difference between length measurements made under the least and the most denaturing conditions as compared to the average of the four conditions. This observation is emphasized by the data presented graphically in Figure 2.

The original intent of this work was to characterize and identify accurate internal standards for use in measuring RNAs of unknown size. Therefore we calculated the ratio of bases per unit length for each nucleic acid species under each spreading condition. These ratios, shown in Table 2, were calculated using the number of bases per molecule as determined by sequence analysis and the electron micrographically determined lengths listed in Table 1.

Two features of this analysis are notable: 1) The RNA molecules have

Figure 2 Length distributions of measured molecules. *E. coli* 16S rRNA and 23S rRNA, Q β RNA and MS2 RNA were prepared for electron microscopy using four denaturing conditions: formamide, formamide-urea, formamide plus glyoxal or formamide-urea plus glyoxal. Grids were prepared and measurements made as described in Methods. Ordinate, number of molecules measured; Abscissa, length in μ m.

an average of 4300 bases per micrometer (μ m) of length whereas the ϕ X174 circular DNA has an average of 3340 bases per μ m. This is an average of 28% more bases, per μ m for RNA than for DNA when data for all four RNA molecules are averaged, and 2) The linear compactness of the RNA molecules varies with the size of the molecule.

Two explanations seemed possible to account for the differences in the mass per unit length ratios of the RNA as compared to the DNA.

First, ϕ X174 DNA is circular and the RNA species measured are linear molecules. It was possible that the differences observed might be due all or in part to the behavior of a circular molecule as compared to a linear molecule in the spreading technique.

To test this possibility, linear full length single-stranded ϕ X174 molecules were generated by restriction enzyme digestion of ϕ X174 RF DNA followed by denaturation in the presence of glyoxal. As shown by the data

TABLE 1

MEAN CONTOUR LENGTH ^a AND STANDARD DEVIATION				
	FORMAMIDE	FORMAMIDE W/GLYOXAL	UREA-FORMAMIDE	UREA-FORMAMIDE W/GLYOXAL
16S rRNA	0.32 $\pm$ 0.04	0.34 $\pm$ 0.04	0.33 $\pm$ 0.04	0.33 $\pm$ 0.04
23S rRNA	0.64 $\pm$ 0.06	0.69 $\pm$ 0.06	0.67 $\pm$ 0.06	0.70 $\pm$ 0.06
MS2 RNA	0.84 $\pm$ 0.06	0.87 $\pm$ 0.06	0.86 $\pm$ 0.06	0.88 $\pm$ 0.07
Q β RNA	1.00 $\pm$ 0.06	1.03 $\pm$ 0.07	1.01 $\pm$ 0.06	1.04 $\pm$ 0.06
CIRCULAR ϕ X174 SS DNA	1.61 $\pm$ 0.07	1.64 $\pm$ 0.07	1.61 $\pm$ 0.07	1.64 $\pm$ 0.07

^a Length measurement are expressed as μ m.

Each RNA value is the average of 200 measurements.

Each DNA value is the average of 400 measurements.

TABLE 2

BASES PER MICROMETER ^a				
	FORMAMIDE	FORMAMIDE W/GLYOXAL	UREA-FORMAMIDE	UREA-FORMAMIDE W/GLYOXAL
16S rRNA	4800	4500	4700	4700
23S rRNA	4500	4200	4300	4100
MS2 RNA	4200	4100	4200	4100
Q β RNA	4220	4090	4180	4050
CIRCULAR ϕ X174 SS DNA	3350	3280	3350	3280

a) Bases per μ m were calculated using the length measurements shown in Table I and the values for bases per molecule as follows: 16S rRNA, 1541 (4); 23S rRNA, 2904 (5); MS2 RNA, 3569 (3); Q β RNA, 4217 (2); ϕ X174 DNA, 5386 (6).

in Table 3, linear ϕ X174 molecules were 4% shorter than circular ϕ X174 molecules. This linear-circular difference accounts for only a small part of the RNA:DNA difference.

A second possibility to account for the observed difference in the base per μ m ratio of RNA and DNA was that the difference in absolute length of the single-stranded molecules contributed to the different values. As indicated by the data in Table 2, the smaller the mass of the RNA molecule, the more linearly compact is its structure. For example, there are 400 more bases per μ m in 16S *E. coli* rRNA than in Q8 RNA. This translates to almost a 10% difference in bases per μ m between RNA molecules varying in size by 270%. The relationship between bases per RNA molecule (absolute size) and bases per unit length for RNA is described graphically in Figure 3. The RNA molecules (upper curve) manifest an inverse relationship between molecular length and bases per unit length. The curve clearly has its greatest slope with small RNA molecules and levels off as molecular weight increases. Between the two rRNA points and the MS2 and Q8 RNA points the slope drops from -0.24 to -0.02, a decrease in absolute value of 93%. Further down the abscissa, changes in bases per μ m with increased bases per molecule become less significant.

The following experiments were done to test the possibility that the different ratio of bases per μ m of RNA and DNA was due to the size of the molecules measured. Linear ϕ X174 DNA molecules in the same size ranges as the RNA molecules measured were generated by cleavage of ϕ X174 RF with the

TABLE 3

MEAN CONTOUR LENGTH AND BASES PER UNIT LENGTH			
ϕ X174 DNA	Bases ^a	Length ^b	Bases/ μ m
ss circular	5386	1.64 $\pm$ 0.07	3280
ss linear	5386	1.58 $\pm$ 0.08	3410
Hpa II fragment A	2748	0.77 $\pm$ 0.07	3600
Hpa II fragment B	1697	0.44 $\pm$ 0.05	3900

a) Bases per ϕ X174 DNA molecule obtained by sequence data (6).

b) Mean length and standard deviation in μ m. Values obtained from 100 measurements of each molecular species.

Figure 3 Relationship of molecule size to bases per unit length. Calculated values of bases per μm are plotted versus molecule size in bases for RNAs (○—○): 1541 bases (16S rRNA), 2904 bases (23S rRNA), 3569 bases (MS2 RNA), 4217 bases (Q8 RNA), ϕX174 DNA (●—●): 1697 bases (ϕX174 Hpa II fragment A), 2748 bases (ϕX174 Hpa II fragment B), 5386 bases (ϕX174 full length linear single strand), and (▲—▲) the linear forms of PBR322 DNA (4361 bases) and M13MP2 DNA (7196 bases). Data plotted is from measurements made using 80% formamide plus glyoxal treatment for both RNA and DNA. Standard error is indicated by vertical bars.

restriction endonuclease HpaII.

The restriction fragments were separated by electrophoresis and denatured to yield single strands as described in Methods. The single strand HpaII fragments of 1697 and 2478 bases were spread using the 80% formamide plus glyoxal treatment. Histograms of the measurements of these two restriction fragments as compared with single strand linear and circular ϕX174 molecules are shown in Figure 4. Two grids were made of each DNA sample and populations of fifty molecules were measured from each grid. The average length measurements of these molecules and the standard deviations for the 100 molecules counted are shown in Table 3 as are the calculated numbers for bases per μm of length. The relationship of molecule size and ratio of bases per μm for DNA was plotted and compared with the data for similarly sized RNA molecules as shown in Figure 3.

The plot of these data shows that DNA (solid symbols) manifests an almost parallel dependence of bases per unit length on molecule size to that shown by RNA (open symbols). The plot of the data generated by the DNA measurements has the same decreasing slope with increasing bases per molecule

Figure 4 Length distributions of measured DNA molecules. ϕ X174 full length single-strand circular (a) and linear (b) DNA and ϕ X174 DNA fragments B (1697 bases) (c) and A (2748 bases) (d) generated by Hpa II cleavage were prepared for electron microscopy by spreading in 80% formamide with glyoxal pretreatment. Grids were prepared and measurements made as described in Methods. Ordinate, number of molecules measured; Abscissa, length in μ m.

as is displayed for RNA. Therefore in both cases, for both RNA and DNA molecules there is an inverse relationship between molecular length and bases per unit length. The slope of this curve decreases as the absolute size of the molecule increases. For molecules having a size of 3000 bases or greater the changes in bases per μ m of length becomes less and less significant as the size of the molecule increases.

We have also measured the single-stranded linear and circular DNAs of the plasmids PBR322 and M13MP2. The size, by sequence analysis of these two DNAs is 4361 and 7196 bases respectively. Our measurements of these DNAs in linear form give a base per unit length ratio of 3550 bases per μ m and 3400 bases per μ m respectively. These values fall on and extend the curve of data obtained with ϕ X174 DNA and the ϕ X174 DNA fragments shown in Figure 3 thereby confirming this data with two other different DNAs.

The second conclusion that can be drawn from this data is that there is a significant ¹³ difference in the base per unit length ratios of DNA and RNA molecules. RNA has an average of 17.5% more bases per μ m of length than DNA. This difference in base per unit length ratio is constant within 1% for all lengths tested when data from similarly sized molecules is compared.

DISCUSSION

The data reported here show that single-stranded RNA has 17.5% more bases per unit length than single-stranded DNA as measured by electron microscopy. This result is observed when molecules of similar size are measured by identical electron microscopic techniques using the same internal standard.

The data reported here also demonstrate that for both RNA and DNA the ratio of the number of bases per unit length varies inversely with the absolute size of the molecule measured. As shown by the data in Figure 3, this variation is greatest when small molecules are compared with large molecules. For example the 1697 base HpaII restriction fragment of ϕ X174 DNA is 11% more compact than the full length linear of ϕ X174 DNA (5386 bases) which is 320% larger. Similarly, there are over 400 more bases per μ m in 16S rRNA (1541 bases) than in Q β RNA (4217 bases). This is a 10% difference in bases per μ m between molecules varying in size by 270%.

Also shown by the data in Figure 3 is the fact that the slope of the curve, that is the change in bases per unit length as a function of absolute size, clearly approaches zero as the size of the molecule increases. The slope decreases by an absolute value of 93%, from -0.24 to -0.02, between the two rRNA values and the values for MS2 and Q β RNAs. Accordingly, as larger molecules are considered, the changes in bases per unit length become less significant. In a dynamic spreading solution molecules are stretched by the forces of diffusion. The more nucleic acid surface available to the diffusive force of the hyperphase solution, the greater the stretch. A large molecule is more readily acted upon and stretched by the hyperphase solution rushing past the nucleic acid barrier than is a small molecule. Our data indicate that small single-stranded molecules (3000 bases or less) are not extended maximally on the hyperphase whereas larger molecules approach the maximal lengths allowed by their molecular structure under these spreading conditions.

The reasons for the difference in the base per unit length ratio of RNA as compared to DNA are not clear. Our finding that RNA is more compact linearly than DNA is consistent with the fact that RNA is denser than DNA as measured by equilibrium density centrifugation. Additionally, Bolton and Kearns (11,12) have proposed a hydrogen bonding scheme which assigns a definite role to the 2' OH in the conformation of the RNA. Based on high resolution paramagnetic resonance (PMR) spectra they conclude that the 2' OH proton in RNA is hydrogen bonded to a water molecule which is correctly located to act as proton donor in a hydrogen bond to the 3' phosphate.

Therefore, this evidence suggests that a water molecule can serve as a hydrogen bonding bridge between the 2' OH of the ribose and the phosphate of the backbone.

Bolton and Kearns conclude (11,12) that this hydrogen bonding in RNA, not found in DNA, can explain both the conformational difference of RNA and the greater thermal stability of RNA. Conceivably this water bridge could add stability to the RNA such that the hydrodynamic forces involved in nucleic acid spreading could not stretch the single-stranded RNA molecule to the same extent as the single-stranded DNA molecule.

Our findings lead us to conclude that the choice of an appropriate internal standard is critical for the accurate determination of the size of single-stranded nucleic acids. Comparison of absolute values previously reported for given single-stranded nucleic acids show wide variation. For example, the 16 and 23S rRNAs have been measured in at least 5 electron microscopic studies prior to this one (14,15,16,17,18) and values reported for the 16S rRNA species vary from $0.33 \pm 0.04 \mu\text{m}$ to $0.54 \pm 0.06 \mu\text{m}$. These differences in absolute measurement are attributed to, in most instances, variation in three factors: microscope calibration, reagent quality (formamide, the principal denaturing reagent in protein film spreading is a labile compound) and spreading conditions and technique. However, by use of judiciously chosen, well-characterized, internal standards, measurements of single-stranded nucleic acids by electron microscopy can have significance.

The data reported here identify two factors that are important in the choice of an appropriate internal standard for electron microscopic analysis of single-strand nucleic acids: 1) DNA and RNA exhibit a significant difference in bases per unit length; therefore, ideally, the homologous nucleic acid should be used as an internal standard in measurements for molecular weight determinations. In instances where it is advantageous to have a circular marker, these differences should be taken into account and appropriate calibrations made, and 2) Since the base per unit length ratio can alter significantly as a function of the absolute size of the molecule, presumably due to the greater hydrodynamic forces exerted on a larger molecule, then it is imperative to use an internal standard that is close in size to the molecule being measured.

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