Atomic force microscopy of reovirus dsRNA: a routine technique for length measurements

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ABSTRACT

Atomic force microscopy (AFM) was used to image reovirus double stranded RNA (dsRNA) deposited from diluted buffer solution onto a chemically treated mica surface. This procedure allows AFM images of dsRNA molecules to be obtained with a quality close to that obtained with conventional electron microscopy. The length of the molecules were measured directly on a computer display using the digitally acquired images. The lengths of the molecules varied between 0.2 and 1.8 μm . Statistical analysis showed a multimodal distribution with clear maxima at 0.4, 0.65 and 1.05 μm . These data are in a good agreement with those obtained by electron microscopy and gel electrophoresis.

INTRODUCTION

Atomic Force Microscopy (AFM) is a relatively new tool (1) which has enormous potential importance to structural biology. It works by measuring tiny contact forces between the surface and a scanning tip. The AFM offers unique advantages in its potential for very high resolution of DNA and RNA (and their complexes with proteins and small ligands) without need of stains, shadows and labels. Furthermore, the instrument can be operated in air or under liquids (2). It is theoretically capable of resolving structural details at the level of atomic dimensions, provided the specimen is dynamically stable and flat. However, an immediate practical limitation to the application of AFM to structural studies of biological macromolecules is sample preparation (3). The macromolecules must be tethered to the substrate surface in order to avoid resolution-limiting motion caused by the sweeping tip during scanning (3). Images of DNA have been obtained by means of drying a DNA solution droplet onto freshly cleaved mica (4). Henderson (5) deposited DNA from a solution which contained MgCl_2 . However, the quality of the DNA images shown in these papers is lower than is normally obtained by electron microscopy. Surface treatments which provide better tethering of the molecule to the substrate have been described recently. An ionic treatment of mica for binding DNA has been described in (6) and (7). This permits reliable imaging in an AFM with a resolution of a few tens of nm. This approach has been

used in (8) to obtain improved resolution by imaging under a covering layer of propanol and using specially manufactured sharp tips. Propanol was used because the DNA was not stable under water (8). We described recently a method for mica modification which is based on treatment of mica with aminopropyltriethoxy silane in very mild conditions (9–12). The procedure has allowed us to obtain reliable images of DNA using commercial instruments with commercial cantilevers. Images of DNA molecules as long as a whole lambda DNA molecule were obtained (11,12). This procedure leads to strong DNA attachment allowing us to image DNA under water as well as in air (12).

In this report, we describe the use of AFM imaging of dsRNA extracted from reovirus as a method for determining its molecular weight distribution. This virus possesses a multi-segment genome, represented by dsRNA molecules of different lengths (13–16). Our AFM images of reovirus dsRNA show that the length distribution of RNA molecules is very close to that obtained by electron microscopy (15,16). The simplicity of the sample preparation procedure for the AFM and fast processing of data (which are digital from the outset) make the technique an attractive alternative to electron microscopy.

MATERIALS AND METHODS

dsRNA samples

Reovirus genome dsRNA was isolated from purified virions using a standard technique (13). Briefly, the virus was disrupted by extraction with phenol and nucleic acids were concentrated by precipitation with ethanol. Genomic dsRNA was purified by gel filtration (14).

Sample preparation for AFM

Freshly cleaved strips of mica were left in a 3-aminopropyltriethoxy silane (APTES) atmosphere created in a 2l glass desiccator under ambient conditions. 30 μl of APTES was placed at the bottom of the desiccator to create the APTES atmosphere. Modified mica strips were immersed into buffer solution containing RNA in 10mM Tris-HCl (pH 7), 10–20 mM NaCl and 5 mM EDTA and incubated at room temperature for 2 hours. The concentration of RNA was 0.1 $\mu\text{g}/\text{ml}$. The samples were

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then rinsed with deionized water (NanoPure water system, Barnstead, 2555 Kerper Blvd. Dubuque, IA), blotted at the edge and vacuum dried.

Apparatus

Imaging was carried out in air on a NanoScope II STM/AFM equipped with a 'D' scanning head (Digital Instruments, Inc., Santa Barbara, CA). The head was calibrated by scanning a diffraction grating. The scanning rate in the experiments was varied between 2.03 Hz for large scanning areas ($4 \times 4 \mu\text{m}$ and more) and 3.55 Hz for smaller areas. Commercial triangular cantilevers were used (from Digital Instruments, with wide legs of 100 and $200 \mu\text{m}$ in length and spring constants of 0.58 and 0.12 N/m respectively).

Data acquisition

AFM data were collected in NanoScope-file format, translated into TIFF-format and transferred to a Macintosh IIfx computer where the lengths of the molecules were measured using the NIH Image 1.40 program. KaledaGraph data analysis software was used for plotting the length distribution data.

RESULTS AND DISCUSSION

AFM imaging of dsRNA

Two typical AFM images of different areas of the same size ($3.2 \mu\text{m}$) are shown in Figs. 1A and 1B. The molecules mostly have a rather convoluted shape and some of them show rather compact geometry. The central part of the area on Fig. 1A was rescanned at higher magnification and the resulting picture ($1.5 \mu\text{m} \times 1.5 \mu\text{m}$ area) is shown in Fig. 1C. Some of the molecules follow a smooth enough geometry to allow their contours to be measured unambiguously. Another area of $2.2 \mu\text{m} \times 2.2 \mu\text{m}$ size is shown in Fig. 2A. The microscope was zoomed onto the center of this picture and results of scanning on the smaller area are shown on Fig. 2B. This picture shows 4 molecules whose size could be measured easily. For example, molecule 1 is $0.37 \mu\text{m}$ in length and molecule 2 is $0.7 \mu\text{m}$. Fig 2C was obtained after repeated scanning over the same area for 20 minutes. This picture is indistinguishable from the previous one and these results demonstrate the stability of RNA molecules under repetitive scanning. Figs. 2B and 2C are non-processed raw data. Instrumental artifacts can give rise to a varying background which

Fig. 1. AFM images of dsRNA from reovirus. Images (A) and (B) show different areas of the same size. Picture (C) was obtained after zooming into the middle of (A). All images were filtered to remove instrumental background variations (see Fig. 2).

Fig. 2. AFM image of $1.8 \mu\text{m}$ area (A). (B), (C) and (D) were obtained from scans over the central part of (A). (C) was recorded after scanning over the area shown in (B) for 20 minutes. (A) (B) and (C) are non-filtered images. (D) is obtained by high pass filtering (B).

Fig. 3. Histograms showing the length distribution of dsRNA molecules. 306 molecules were measured. (A) is raw data and (B) is a histogram obtained after averaging data in $0.05 \mu\text{m}$ intervals.

can be removed by using a high-pass filter (10,11). Results of the high-pass filtering are shown on Fig. 2D. In this image, the upper and lower strand of the loop in molecule 2 are easily recognized. The concrete topology of intersection has no effect on length measurements, but the possibility of recognition of such topological peculiarities is very important for studies of RNA and DNA higher order structure, for example, supercoiled DNA.

Statistics of dsRNA length measurements

AFM images similar to those in Figs. 1A and 1B were used for measuring dsRNA molecule lengths. Results of measurements for a set of more than 300 molecules are shown as a histogram in Fig. 3 A. AFM images of $4 \times 4 \mu\text{m}$ scanning area typically contained 20–30 well separated molecules. The images for the entire data set were collected during one hour of AFM scanning. The histogram in Fig. 3A contains three major peaks which are most clearly seen after smoothing the initial distribution shown in Fig. 3A. Figure 3B shows the data after binning with a $0.05 \mu\text{m}$ slot-width using the KaleidaGraph software. Three major peaks with maxima around 0.4, 0.65 and $1.05 \mu\text{m}$ are clearly seen in both the raw data and the smoothed histogram. We tried many selections for the bin-width, finding that they had little effect on the shape of the distribution.

Extracted dsRNA of reovirus is in a segmented form. Evidence for segmentation of reovirus dsRNA was first obtained from electron microscopic studies (15,16). Fragments of dsRNA fell into three size classes centered around $0.38 \pm 0.09 \mu\text{m}$, $0.64 \pm 0.26 \mu\text{m}$ and $1.10 \pm 0.27 \mu\text{m}$. These data are shown as bars on our histograms (Fig. 3A and 3B). The electron microscopic data are very close to those obtained in this work. It is possible to resolve the reovirus dsRNA into 10 distinct pieces using polyacrylamide gel electrophoresis. They fall into 3 classes, small, medium and large with mean sizes in agreement with the results quoted above (17). Our sample is too small for each of the 10 fragments to show up distinctly in the raw data, however, the resolution of the histograms obtained in this work is similar to that obtained in electron microscopic studies (15,16).

CONCLUSIONS

The method of sample preparation we have described can be used for routine AFM analysis of nucleic acids. At the moment its resolution of both lengths and widths is approximately the same as conventional electron microscopy. However, the AFM has some advantages. The sample preparation procedure is simple and special procedures to prevent sample degradation (15, 16) are not required. Second, samples can be prepared by deposition directly from salt solution, allowing variations of salt concentration and incubation temperature. We have found that the procedure works for incubation temperatures between 0 and 60°C . Third, stable binding of the sample to the substrate allows structural studies to be performed not only in air, but also under water and, presumably, in buffer solution. These unique features are very important for studies of such 'fragile' samples as nucleoproteins. Fourth, the resolution of the AFM depends on tip geometry (2), and recent advances in the tip manufacturing technology give grounds for optimism.

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REFERENCES

1. Binnig, G., Quate, C.F. and Gerber, Ch. (1986) *Phys. Rev. Lett.* **56**, 930–933.
2. Drake, B., Prater, C.B., Weisenhorn, A.L., Gould, S.A.C., Albrecht, T.R., Quate, C.F., Cannell, D.S., Hansma, H.G. and Hansma, P.K. (1989) *Science* **243**, 1586–1589.
3. Lindsay, S.M., In J. Bonnell (ed.), *Scanning Tunneling Microscopy: Theory, Techniques and Applications*. VCH Press, **in press**.
4. Thundat, T., Warmack, R. J., Allison, D. P., Lourenco, A. J., and Ferrel, T. L. *J. Vac. Sci. Technol.* (in press).
5. Henderson, E. (1992) *Nucl. Acids Res.* **20**, 445–447.
6. Bustamante, C., Vesenska, J., Tang, C.L., Rees, W., Gutfold, M. and Keller, R. *Biochemistry* **31**, 22–26 (1992).
7. Vesenska, J., Tang, C.L., Gutfold, M., Keller, D. and Bustamante, C. *Ultramicroscopy*, **in press**.
8. Hansma, H.G., Vesenska, J., Seigerist, C., Kelderman, G., Morett, H., Sinsheimer, R.L., Eilings, V., Bustamante, C. and Hansma, P.K. (1992) *Science* **256**, 1180–1184.
9. Lyubchenko, Y.L., Lindsay, S.M., Gall, A. A., Shlyakhtenko, L.S., Harrington, R.E. (1991) *Biophys. J.* **61** A149.
10. Lindsay, S.M., Lyubchenko, Y.L., Gall, A.A., Shlyakhtenko, L.S. and Harrington, R.E. (1992) *SPIE Proc. Internat. Symp. Laser Spectr.*, Los Angeles **in press**.
11. Lyubchenko, Y. L., Shlyakhtenko, L. S., Harrington, R.E., Oden, P.I. and Lindsay, S.M. (1992) *Proc. Natl. Acad. Sci., USA*, **in press**.
12. Lyubchenko, Y. L., Shlyakhtenko, L.S., Gall, A. A., Harrington, R. E., Oden, P.I., Jacobs, B. L. and Lindsay, S. M. (1992) *J. Biomol. Struct. Dyn.* **in press**.
13. Ramig, R.F., Cross, R.K. and Fields, B.N. (1977) *J. Virol.* **22**, 726–733.
14. Langland, J.O., Pettiford, S.M. and Jacobs, B.L., **submitted**.
15. Gomatos, P. J. and Stoeckenius, W. (1964) *Proc. Natl. Acad. Sci., USA* **52**, 1449–1455.
16. Vasquez, C. and Kleinschmidt, A. K. (1968) *J. Mol. Biol.* **34**, 137–147.
17. Shatkin, A.J., Sipe, J.D. and Lon, P. (1968) *J. Virology* **2**, 986–991.