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The measurement of exonuclease activities by atomic force microscopy

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Abstract We have applied atomic force microscopy (AFM) to the measurement of BAL 31 nuclease activities. BAL 31 nuclease, a species of exonuclease, is used to remove unwanted sequences from the termini of DNA before cloning. For cutting out only the appropriate sequences, it is important to know the nuclease properties, such as digestion speed and the distribution of the lengths of the digested DNA. AFM was used to obtain accurate measurements on the lengths of DNA fragments before and after BAL 31 nuclease digestion. We analyzed 4 DNAs with known number of base pairs (288, 778, 1818, and 3162 base pairs) for correlating the contour length measured by AFM with the number of base pairs under the deposition conditions used. We used this calibration for analyzing DNA degradation by BAL 31 nuclease from the AFM measurement of contour lengths of digested DNAs. In addition, the distribution of digested DNA could be analyzed in more detail by AFM than by electrophoresis, because digested DNA were measured as a population by electrophoresis, but were measured individually by AFM. These results show that AFM will be a useful new technique for measuring nuclease activities.

Key words DNA · Enzyme activity · Atomic force microscopy · Exonuclease · BAL 31 nuclease

Introduction

Atomic force microscopy (AFM) (Binnig et al. 1986; Rugar and Hansma 1990) is a useful technique for reliably imaging biomolecules on a nanometer scale under air or liquid conditions without complicated sample preparation techniques, such as staining, used in transmission electron microscopy (Coggins 1987). For instance, DNA (Allen et al. 1993; Bustamante et al. 1993; Hansma et al. 1992, 1993, 1994, 1995; Lyubchenko et al. 1993; Thundat et al. 1992), protein (Hallet et al. 1995) and the interaction of DNA and protein (Bezanilla et al. 1994; Erie et al. 1994; Fritzche et al. 1995; Hansma et al. 1995; Rees et al. 1993; Wyman et al. 1995) have been observed. Thus AFM is expected to be a powerful new tool for investigating microscopic structures in molecular biology.

AFM images of DNA are larger than the real structure because the images are created by the convolution of the DNA itself and the radius of the curvature of a tip. However, the effect is negligible for length measurements because the length is much greater than the curvature of the tip, and AFM is therefore suitable for these measurements. Thus, the measurement of contour lengths of DNA molecules has been performed in a variety of environments (air, propanol, aqueous solutions) with different fixation techniques (Bustamante et al. 1992; Coury et al. 1995; Hansma et al. 1996; Murray et al. 1993; Muzzalupo et al. 1995; Pietrasanta et al. 1994; Schaper et al. 1993, 1994; Shaiu et al. 1993; Thundat et al. 1993, 1994; Vesenka et al. 1992; Yang et al. 1992; Zenhausem et al. 1992).

As an exonuclease digests DNA from the termini, the length of the DNA is a trace of the exonuclease reaction. Thus we can measure the activity of an exonuclease by measuring the contour lengths of digested DNA fragments. BAL 31 nuclease, a species of exonuclease, can be used to remove unwanted sequences, from the termini of DNA before cloning (Sambrook et al. 1989). However, it is not easy to remove only the unwanted sequences, for the following reasons. First, BAL 31 nuclease degrades AT-rich sequences significantly more rapidly than it degrades GC-rich regions. Second, BAL 31 nuclease contains two kinetically distinct forms (Wei et al. 1983), a fast and a slow form: the slow form is a proteolytic degradation product of the fast form. These properties would cause unexpected digestion of DNA and precise mesurements of BAL 31 nuclease activity will be needed.

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We applied AFM to the measurement of BAL 31 nuclease activity because of the better accuracy of the length measurements. However, AFM cannot measure the number of base pairs directly. It is more important to determine the number of base pairs of the unknown DNA than only to know the contour length, because the sequences removed by BAL 31 nuclease are usually shown by the number of base pairs. Therefore, a standard curve representing the correlation between the measured length of a DNA fragment and the number of base pairs will be needed.

In order to obtain a standard curve for relating the number of base pairs to the measured contour lengths, we made a series of linear DNA fragments with a known number of base pairs from 278 bp to 3162 bp, fixed them on mica substrates, and measured the lengths with AFM. We then used this standard curve for determining the base pair numbers of DNA digested with BAL 31 nuclease.

Experimental

Nucleic acids

Linear DNA fragments of 288, 778, 1818, and 3162 base pairs for length measurements were made as follows. 3162 bp DNA fragments were made by linearization of plasmid pUC 118 with *Eco*RI in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. 1818 bp DNA fragments were isolated from *Mlu*I digests of plasmid pU-1.8 k which was created by inserting the *Bgl*I fragment (3480-929) of pBR 322, the ligated synthetic oligonucleotide linker containing the *Mlu*I site, into pUC 118 (TAKARA SHUZO CO., LTD, JP) at the *Sma*I site. 778 bp DNA fragments were isolated from *Eco*RII-*Xba*I digests of plasmid pUC-GFP, which was created by inserting the *Bam*HI-*Bcl*I fragment of the GFP DNA in pGFP (CLONTECH Laboratories, Inc. CA) into pUC 118 at the *Bam*HI site. These plasmid DNAs were isolated from *E. coli* JM 109 and purified using Qiagen tip-25 columns (Qiagen Inc. CA) according to the manufacturer's instructions. 228 bp DNA fragments were created by amplification of nucleotides 728–1014 of GFP DNA by PCR, using the primers GFPF (5′-GTTGAATTAGATGGTGATGT) and GFPR (5′-TCAGCACGTGTCTTGTAGTT) at 50 °C with Taq polymerase (TOYOBO CO., LTD, JP) according to the manufacturer's instructions.

Restriction fragments (778 and 1818 bp fragments), and PCR products (288 bp) were separated by electrophoresis through an agarose gel, and recovered from the gel by electroelution into dialysis bags.

Digestion of linear pUC 118 DNA by BAL 31 nuclease

Linear pUC 118 DNA (5 µg) was digested with BAL 31 nuclease (6 units) in 20 mM Tris-HCl (pH 7.2), 600 mM NaCl, 12.5 mM $CaCl_2$, 12.5 mM $MgCl_2$, 1 mM EDTA at 30 °C. The digestion was terminated by extraction with phenol : chloroform.

DNA deposition on a mica substrate

10 µl each of linear DNA and DNA digested with BAL 31 nuclease, diluted to 0.01 µg/ml with 10 mm HEPES, 10 mM $MgCl_2$, were deposited directly on freshly cleaved mica for 30 minutes. The samples were then rinsed with 3 ml of MilliQ (Millipore Corp., MA) purified water, and dried under argon gas flow. All samples were stored in a vacuum desiccator until AFM imaging.

Agarose gel electrophoresis

DNA was electrophoresed in 1% agarose gels in Tris-borate-EDTA buffer (pH 8.2), and the DNA band was visualized by UV light after staining with ethidium bromide.

AFM

AFM imaging was performed in tapping mode in air with the Nanoscope IIIa (Digital Instruments, Santa Barbara, CA). The E scanner, with 5% accuracy based on measurements of imaging a 1 µm pitch grating standard supplied by the manufacturer, was used for all samples. The silicon cantilevers, 125 µm long and typically about 13–100 N/m in spring constant, were used for the tapping mode imaging. All images presented in this paper are raw data except for flattening.

The contour lengths of DNA were measured with NIH Image (National Institute of Health, MD).

Results

Length measurements of a series of linear DNA fragments

Figure 1 shows typical images of linear DNA fragments of different lengths. In the image of the 3162 bp DNA (A), many fragments are of the same length, except for small debris and a small number of short fragments. The short fragments and small debris may derived from contamination as we did not purify the linearized pUC 118 plasmids in sample preparation. Therefore, we did not include these

Table 1 Length measurements of 3162, 1818, 778, and 288 bp DNA

Base pairs	*n*	mean (nm)	SD (nm)	Calculated base spacing (nm)
3162	83	1087	32	0.344
1818	17	636	17	0.350
778	11	260	17	0.335
288	18	88	9	0.307

Base spacing was calculated by division of the average length by the number of base pairs

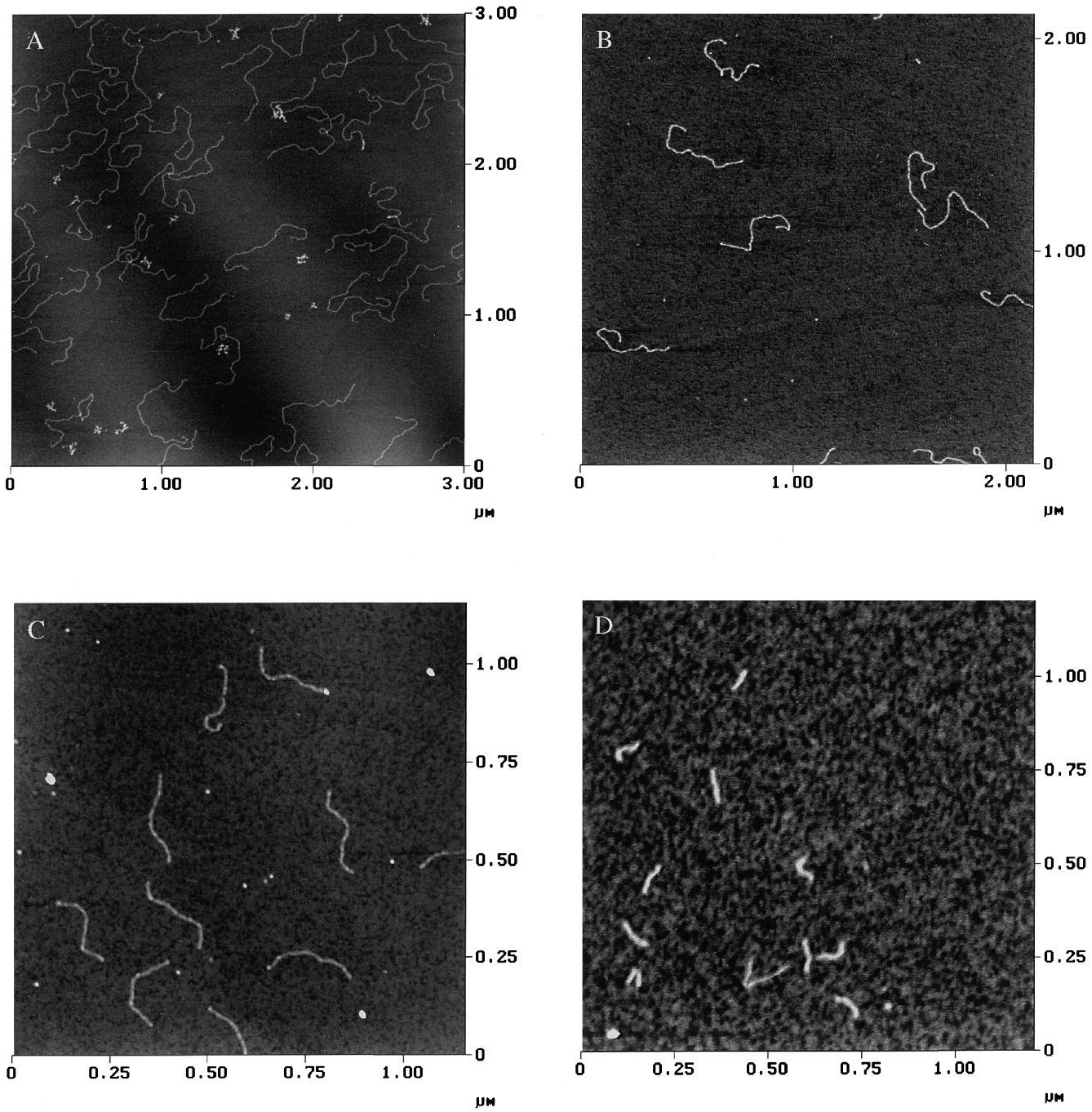

Fig. 1 AFM images of linear DNA fragments of (**A**) 3162 bp, (**B**) 1818 bp, (**C**) 778 bp and (**D**) 288 bp. DNA fragments were fixed on a cleaved mica surface using Mg^{2+}, and imaged using the tapping mode in air

data in the statistical calculation. In all other images of 288 bp, 778 bp, and 1818 bp DNA fragments, we obtained a nearly uniform length. The average length and standard deviation of 288, 778, 1818 and 3162 bp DNA fragments were 88±9 nm, 260±17 nm, 636±17 nm and 1087±32 nm, respectively (Table 1). The regression line of measured contour length against the number of base pairs (Fig. 2) shows good linearity, and the value of the slope is 0.346 nm/bp, which is consistent with the spacing in B-form DNA (0.338 nm/bp) (Saenger 1984)

Measurements of the length and the distributions of DNA digested by BAL 31 nuclease

We used 3162 bp DNA fragments, linearized pUC118 plasmid DNAs, as a control, with the same image and length distribution as described previously. Figure 3 shows the AFM images of nuclease digested DNA fragments. In

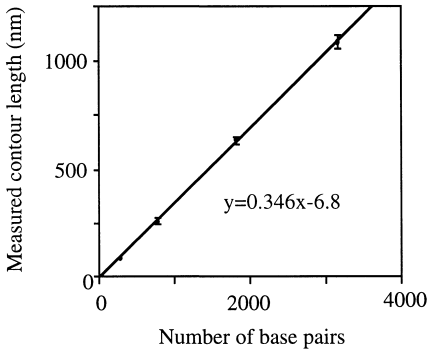

Fig. 2 The average length of each linear DNA fragment plotted against the number of base pairs, and the regression analysis. The error bar represents the standard deviation

Table 2 Length measurements of 3162 bp DNA fragments digested by BAL 31 nuclease

Digestion time (min)	AFM			Gel electrophoresis
	Average length (nm)	Standard deviation (nm)	Estimated base pairs [a]	Estimated base pairs
0	1087	32	3161±92	3250
1	947	37	2757±107	3025
2.5	794	61	2314±176	2600
5	668	96	1950±277	2050

The estimation of the number of base pairs in AFM measurements was calculated from the standard curve (Fig. 1). In electrophoresis, it was obtained from the logarithmic plot of the number of base pairs of standard samples plotted against the migration. Each value of estimated pairs is represented by [a] (mean±SD)

the image of 1-minute digested DNA (Fig. 3 A), many fragments were found to be of same length, except for a few short fragments. The short fragments occur more frequently in 2.5-minute digested samples than in 1-minute digested samples. These short fragments had almost disappeared in the 5-minute digested samples. The reason for the appearance of the short fragments will be discussed later. The short fragments are outside the main distribution shown in Fig. 4 and we did not include them in the calculations. The average length and standard deviation of the main peak for control, 1-, 2.5- and 5-minute digested samples were 1087±32 nm, 947±37 nm, 794±61 nm and 668±96 nm, respectively.

Table 2 shows the calculated number of base pairs from the calibration curve and estimated values obtained from the electrophoresis used to verify that the number of base pairs obtained with AFM was correct. These results show that AFM values were in agreement with the values obtained by electrophoresis.

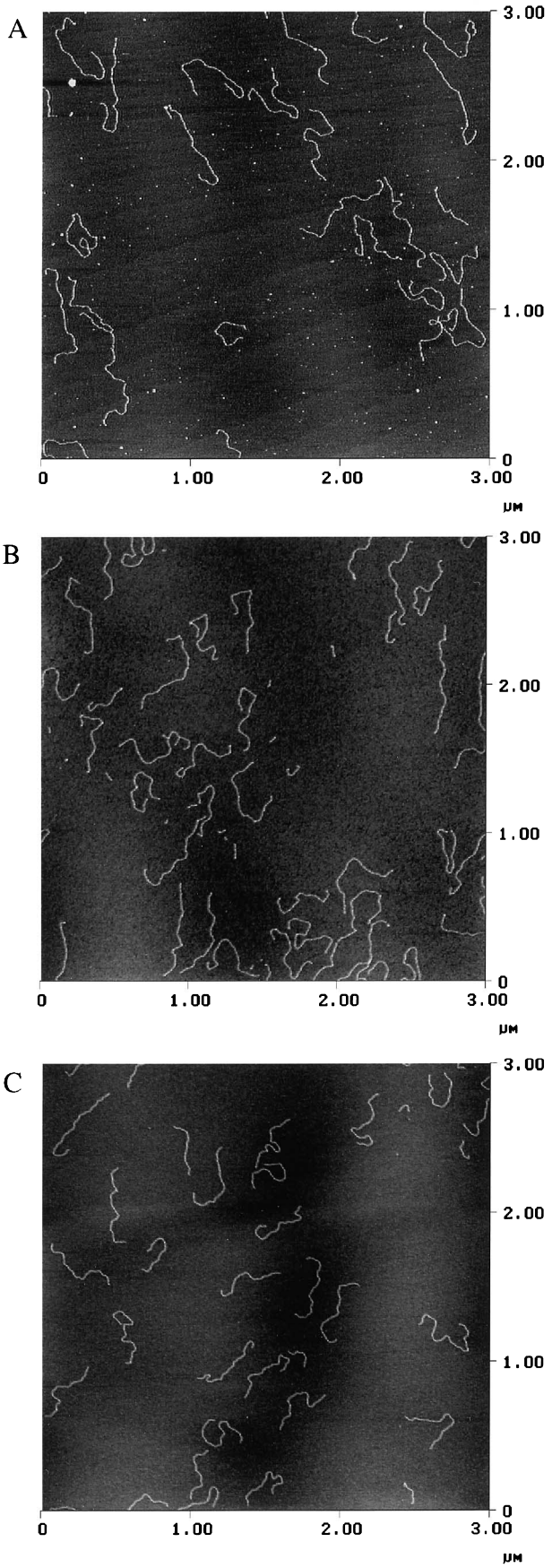

Fig. 3 AFM images of DNA fragments for (**A**) 1-, (**B**) 2.5- and (**C**) 5-minute BAL 31 nuclease digestions. Samples were fixed on a mica substrate. The reaction of the nuclease was terminated by extraction with phenol : chloroform

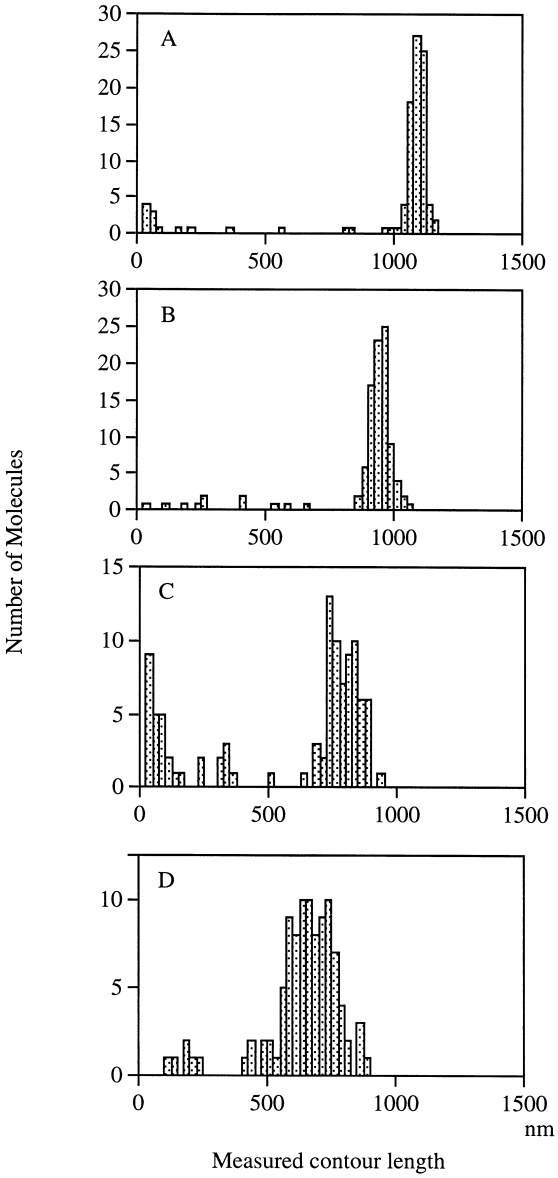

Fig. 4 A–D The length distribution of digested DNA fragments by BAL 31 nuclease; (**A**) control, (**B**) 1-minute, (**C**) 2.5-minute and (**D**) 5-minute digested DNA

Discussion

Measurement of DNA length

Measurements of the contour length of DNA have previously been performed in order to determine whether DNAs take the dehydrated A-form or hydrated B-form. In the most of these reports, circular DNA, which is easy to discriminate from the artifacts in AFM images, or very short fragments (<200 bp) were used. However, there are no data which address the correlation between the measured length and the number of base pairs over a wide range from short fragments to long ones. We measured the contour lengths of linear DNA from 288 bp to 3162 bp, and performed a regression analysis. The average spacing between base pairs of 288 bp DNA is 0.306±0.03 nm, which is between the length of A-, and B-form DNA. It was reported that DNA with a length shorter than 200 bp, was imaged as the A-form (Hansma et al. 1996). They suggested that shorter DNA fragments might have fewer sites to bind the mica surface than long DNA and that short fragments would shrink to A-form DNA in a dehydrated environment, while long DNA would hold its B-form length. DNA of 288 bp might be too long to convert from B-form to A-form. The SD value of 288 bp DNA fragments was 9 nm, which corresponds to the tip radius we used. Taking account of this SD value, the spacing of 288 bp DNA covers the length of A- and B-form DNA. Thus we cannot neglect the tip effect in the measurements of short DNA fragments and it would be necessary to use sharper tips to clarify whether a DNA fragment as short as 288 bp is A- or B-form.

From 778 bp DNA to 3162 bp DNA, the spacing of base pairs ranged from 0.33 nm to 0.35 nm, and the regression coefficient was 0.344 nm/bp, consistent with B-form DNA spacing. As described above, the length measurements of DNA by AFM have previously been performed using plasmids and short fragments of DNA with fewer than 200 base pairs. Thus our results have extended the range of previous studies.

Measurement of the length and the distributions of DNA digested by BAL 31 nuclease

Short fragments appearing in the images of 1- and 2.5-minute digested DNA were outside the main area of each histogram. There may be two possibilities for the appearance of these short fragments. First, they could be the consequence of the digestion of small debris and short fragments contained in the control samples. Second, BAL 31 nuclease could cut out control DNA randomly at the nick points, which would produce small fragments. The standard deviation in the main peak of 1-minute digested samples was 107 bp, almost the same as the 92 bp value of the control sample. The standard deviation increased considerably to 176 bp and 277 bp in 2.5-minute and 5-minute samples, respectively. These increases in SD values suggest that the reaction of BAL 31 nuclease is distributive. This result means that long reaction times cause expansion of the length distribution of digested DNAs. Therefore, short reaction time are preferable if BAL 31 nuclease is to be used to cut out only unwanted sequences in sample preparation for DNA cloning.

Conclusion

In this paper, we have measured the contour length of DNA, clarified the correlation between the length and the number of base pairs, and obtained a standard curve. By using the standard curve we have successfully measured the dis-

tribution of DNA digested with BAL 31 nuclease. As AFM can measure the lengths of individual DNA fragments, the distribution obtained by AFM is better than that obtained by electrophoresis. In addition, as AFM needs only 100 pg of DNA it is a very effective tool for the detailed analysis of small amounts of DNA.

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