The Folded State of Long Duplex-DNA Chain Reflects Its Solution History

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ABSTRACT The higher-order structure of compacted single giant DNA induced by complexation with polypeptide (poly-Arg) in NaCl solution was investigated using fluorescence microscopy. As the poly-Arg concentration increased, the mean size of extended DNA chains gradually decreased. In the presence of excess poly-Arg, individual DNA chains collapsed into compact globules, and the degree of collapse of the DNA chains depended not only on the concentration of poly-Arg, but also on the time course of the addition of poly-Arg and NaCl, indicating that the structure of the collapsed DNA is not determined simply according to the minimum free energy. We discuss theoretically the presence of multiple-stationary states based on a consideration of simple kinetics in the process of binding. Depending on the past history, the number of poly-Arg and Na^+ that bind to each DNA changes markedly. This interesting characteristic of long DNA is discussed in relation to the possible mechanism of self-regulation of gene expression in living cells.

INTRODUCTION

In living prokaryote and eukaryote cells, individual double-stranded DNA chains usually exist in a highly compacted state. For example, in mammalian cells, individual DNA chains have a contour length or full-stretch length, L, on the order of a centimeter (Watson et al., 1987). Because the persistence length λ of a DNA chain is usually on the order of 500 Å (Hargerman, 1988), a DNA chain with $L = 1$ cm corresponds to a linear, freely jointed polymer chain with $N \sim 10^5$ segments, where $N = L/(2\lambda)$. If DNA chains act as ideal linear polymers, the average end-to-end distance is $\langle R \rangle \sim 2\lambda N^{1/2} = \sim 30$ μm. In fact, however, in actual living cells, a long DNA chain is compacted into a small space on the order of 1 μm, indicating that DNA chains are more tightly compacted than an ideal chain. In the present article, we refer to a DNA chain that is significantly compacted compared with the ideal chain as globule DNA. On the other hand, if a purified DNA chain is dissolved in a typical aqueous solution, it swells more than the ideal polymer chain, because of the excluded volume effect between the segments in the DNA chain. We refer to this elongated form as coil DNA. It should be noted that our use of the term "coil" does not refer to the denatured random structure in single-stranded DNA, and that both the coil and globule DNAs preserve their double-stranded structure. Upon coarse graining of the double-stranded structures, individual long DNA chains appear as either elongated coils or compacted globules.

Among globule DNAs in vivo, various types of higher-order structure have been reported. In some viruses, long DNA chains are highly packed and exhibit a liquid crystalline-like structure (Earnshaw and Casjens, 1980). In eukaryote cells, DNA chains are compacted to form a chromatin structure. It is well known that the morphology of the compacted DNA molecule changes drastically depending on the stage in the cell cycle. Thus, a detailed understanding of the manner of packing in long DNA chains is important in biological science. However, despite this importance, our understanding of the globule, or compacted, DNA structure in an aqueous environment remains at a primitive level. This may be due to the lack of a suitable experimental methodology for investigating the higher-order structure of single giant DNA chains in an aqueous environment.

Most previous studies on the higher-order structure of long DNA chains in vitro have been carried out for an ensemble of DNA chains, using experimental tools such as light-scattering, sedimentation, and viscosity. For example, studies on so-called ψ-condensation (Lerman, 1971, 1973), polymer- and salt-induced condensation, have frequently been carried out for the aggregate, or the condensate, of DNAs. Condensation of DNAs induced by various kinds of cationic species have been studied extensively (Arscott et al., 1990; Plum et al., 1990; Bloomfield, 1991). In living cells, because individual DNA chains are compacted independently without knotting to each other, accurate discrimination between single-chain compaction and condensation of multiple DNA chains is essential for obtaining clear insight into the genetic function of long DNA.

Recently, we found that individual long DNA chains undergo a discrete transition between coil and globule states with the addition of various kinds of condensation reagents, such as multivalent cation (Yoshikawa and Yoshikawa, 1995; Yoshikawa et al., 1996a,b), neutral flexible polymer (Minagawa et al., 1994; Vasilevskaya et al., 1995), alcohol (Ueda and Yoshikawa, submitted for publication), and cationic surfactant (Mel'nikov et al., 1995). These findings were obtained by single-chain observation using fluorescence microscopy. The significance of our recent finding is that the transition is first order on the level of single DNA chain, whereas the transition is steep but continuous on the level of ensemble of DNAs (Yoshikawa et al., 1996b). This

Received for publication 3 January 1996 and in final form 22 April 1996.
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0006-3495/96/08/932/08 $2.00

clarifies why there has been essentially no experimental report on the discrete transition in the higher-order structure of DNA. With single-chain observation by fluorescence microscopy, single DNA chains have been shown to undergo a process of nucleation and growth (Yoshikawa and Matsuzawa, 1995), which confirms that the coil-globule transition follows first-order phase transition. The products of DNA compaction induced through the process of nucleation and growth exhibit various shapes, such as toroid and rod, which are considered single molecular crystals (Yoshikawa et al., 1996a). However, the relationship between the steric structure of compacted DNA and the time course of compaction has not yet been clarified, despite its importance in biological science. Therefore, we have extended our study of higher-order structure of DNA to address the following problems: What is the fundamental principle that controls the steric structure of compacted DNA in an aqueous environment in vitro and in vivo ? Does a long DNA chain reflect its past history in its compacted structure?

To investigate the effect of the order dependence of a multistep procedure on the collapse process of long duplex-DNA, we observed the solution structure of DNA/poly-Arginine complex. It has become clear that the folding of single DNA can reflect its past history. We discuss such hysteresis effect with the aid of time-dependent nonlinear differential equation.

MATERIALS AND METHODS

Sample solutions

Bacteriophage T4DNA and poly-L-Arginine (poly-Arg) with an average degree of polymerization of 236 were purchased from Nippon Gene (Tokyo, Japan) and Sigma Chemicals (St. Louis, MO), respectively. T4DNA is composed of 166 kbp, and has a contour length of 56 μm in aqueous solution (Yoshikawa and Matsuzawa, 1995). We used Tris-Borate buffer (45 mM Tris and 45 mM Borate) throughout the experiments. The buffer solution was purified by Millipore filter before measurement. The fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Wako Pure Chemicals (Oosaka, Japan).

Fluorescence microscopy

The complex of a single T4DNA with poly-Arg was observed using fluorescence microscopy. We added 0.3 μM DAPI to the DNA solution (0.3 μM in phosphate), to stain the DNA molecules. Under this condition, the ratio of DAPI molecules bound to DNA is estimated to be 1 per 20 base pairs (Matsuzawa and Yoshikawa, 1994). It has been previously confirmed that the presence of DAPI at this concentration has no significant effect on the persistence length or the contour length of DNA. In addition, under this ultradilute condition of DNA, there is almost no chance for the DNA molecules to collide with each other during the observation period of several minutes.

Sample solution was situated between two thin glass plates (Matsunami No. 1, thickness:120 μm~170 μm) at a wide depth of ~150 μm using spacer glass plates. Because we used a sample depth greater than that in usual fluorescence measurement, many DNA molecules remained in the bulk solution during the observation period. It is noted that the mean size of T4DNA molecules is less than 3 μm. Thus, we could observe the higher-order structure just of the complex in bulk solution that hardly has interaction with glass surface, discounting the images of DNAs absorbed onto the glass surface.

Fluorescence images of the complex were recorded on videotape and processed using an image-processor Argus 50 (Hamamatsu Photonics, Hamamatsu, Japan). Long-axis length was measured on 50~100 randomly chosen complexes under fixed conditions, where the long-axis length is defined as the longest distance in the fluorescence image (Fig. 1).

Electron microscopy

Electron microscopic observation of the complex was carried out by the negative-staining method with an electron microscope JEM1200EX (JEOL, Tokyo, Japan). Carbon-coated copper grids were treated with glow discharge. The grid was floated on a droplet of sample solution (20 μl) for 2 min, drained on filter paper, and finally stained for 30 s with an aqueous solution of 1% uranyl acetate.

RESULTS

Fig. 2 shows the average long-axis length of fluorescent images as a function of the poly-Arg concentration. We measured the length with two different time-dependent procedures.

Route A: Poly-Arg and then sodium chloride were added to the DNA solution.

Route B: Sodium chloride and then poly-Arg were added to the DNA solution.

There was no difference in the final concentrations of poly-Arg and sodium chloride (40 mM) between Route A and Route B. For both routes, the individual DNA chains tended to shrink with an increase in the poly-Arg concentration. This gradual change is in contrast to the discrete behavior in the coil-globule transition using other condensation agents, such as polyamine (Yoshikawa et al., 1996b), hexamine cobalt(III) (Yoshikawa et al., 1996a), polyethylene glycol (Minagawa et al., 1994), and cationic surfactant (Mel'nikov et al., 1995). Fig. 2 indicates that there is a definite difference in the conformation of the complex depending on the time course of the preparation. The system in Fig. 2 is expected to attain a stationary state, because it remains essentially the same even 1 week after the sample preparation.

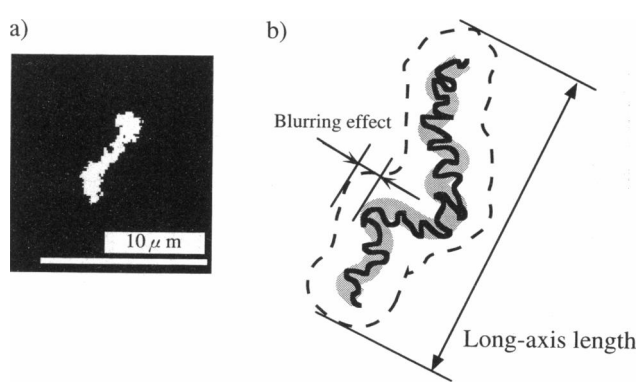

FIGURE 1 (*a*) Fluorescence image of a random coil T4DNA molecule in aqueous solution. (*b*) Schematic diagram of the blurring effect on the fluorescence image. Long-axis length is defined as the longest distance in the image.

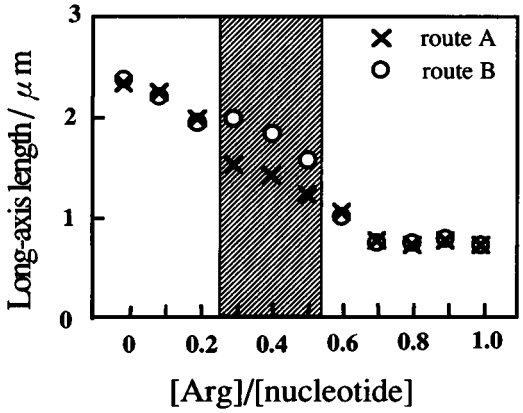

FIGURE 2 Average long-axis length of T4DNA as a function of the poly-Arg concentration in stationary states. The shaded area indicates the region with a distinct difference between routes A and B. The concentrations, [Arg] and [nucleotide], are the monomer concentrations of poly-Arg and DNA. [nucleotide] = 0.3 μM, [NaCl] = 40 mM.

Fig. 3 *a* shows the experimental distribution of the long-axis length for DNA chains obtained through routes A and B, in which individual samples were measured after being allowed to stand for 3 days at 4°C. We have confirmed that the profile of this distribution remains almost constant even after 7 days (data not shown), indicating that the results in Fig. 3 reflect a stationary state. As shown in the difference between the distributions, the complex formed through route A is folded more closely than that formed via route B.

To verify the presence of differences in the distributions with the two routes, we performed a statistical analysis using a rank sum test. Note that the distribution of the long-axis length of the complex should deviate widely from a normal distribution, because of the heterogeneity of the

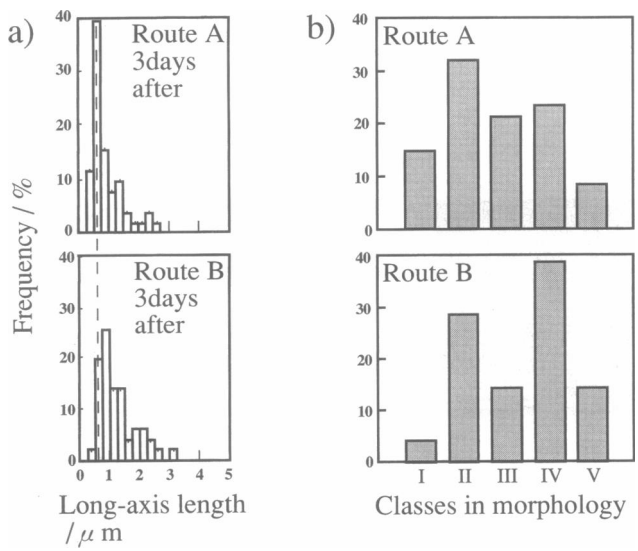

FIGURE 3 (*a*) Difference in the distribution of the long-axis length of the complex at [Arg]/[nucleotide] = 0.3 in route A. (*b*) Difference in the morphology in routes A and B according to the classification given in Fig. 4.

number of poly-Arg bound to DNA. Therefore, we used the Mann-Whitney-Wilcoxon rank sum test (Mann and Whitney, 1947) as a static nonparametric analysis, in which the null hypothesis to be examined is that the samples to be compared are randomly chosen from the same population distribution. The approximate normal deviate Z was 2.93, suggesting that the null hypothesis is rejected at the 5% level; i.e., the two distributions have a statistically significant difference in the shaded region in Fig. 2. On the other hand, for poly-Arg concentrations between 0.6 and 1.0 and between 0 and 0.2, no meaningful difference was recognized between the two different routes.

These results indicate that the shape of the DNA complex is dependent on the sequence of sample preparation. The most plausible explanation for this phenomenon involves the difference in the number of poly-Arg bound to DNA between routes A and B.

Next, we tried to perform an analysis by classifying the profile of the fluorescence intensity of the images of individual DNAs (Fig. 4). Class I includes fluorescence images with a compact bright light spot. This class has one sharp peak of fluorescence intensity within the image of a DNA chain. Class II corresponds to the images with slight loosening from the tightly compacted state having a single maximum and a small tail on the light-intensity distribution. Class III corresponds to the images with several maxima in the fluorescence-intensity distribution. Class IV includes images revealing the shape of thick filaments with a rather broad intensity distribution without intense maximum on the image. Finally, Class V corresponds to the elongated coil state. This class has no fluorescence peak within the image. The distribution of DNA images in each class is shown in Fig. 3 *b*, indicating that route A tends to enhance the collapse of the DNA chain more than route B.

To obtain information regarding structural details from the fluorescence microscopic shapes of compacted DNA, we observed the complexes by transmission electron microscopy (TEM). Fig. 5 shows the TEM image of a globular complex formed in the presence of excess poly-Arg: [Arg]/[nucleotide] = 3.0. We found that DNA globules induced by complexation with poly-Arg exhibited an amorphous-like state. Unfortunately, from the TEM measurement, it was very difficult to study the structural change of DNA chain in relation to the poly-Arg concentration and also to the time-dependent procedure on the sample preparation (hysteresis effect).

DISCUSSION

The results in the present study indicate that the manner of packing of long DNA chains is determined by kinetics, rather than by the principle of the minimum free energy. In the following we theoretically discuss the mechanism how the observed difference depends on the time-dependent procedure (Fig. 3), based on a simple mathematical model.

Let us discuss the effects of kinetics on the higher-order structure in the competitive binding of two cationic

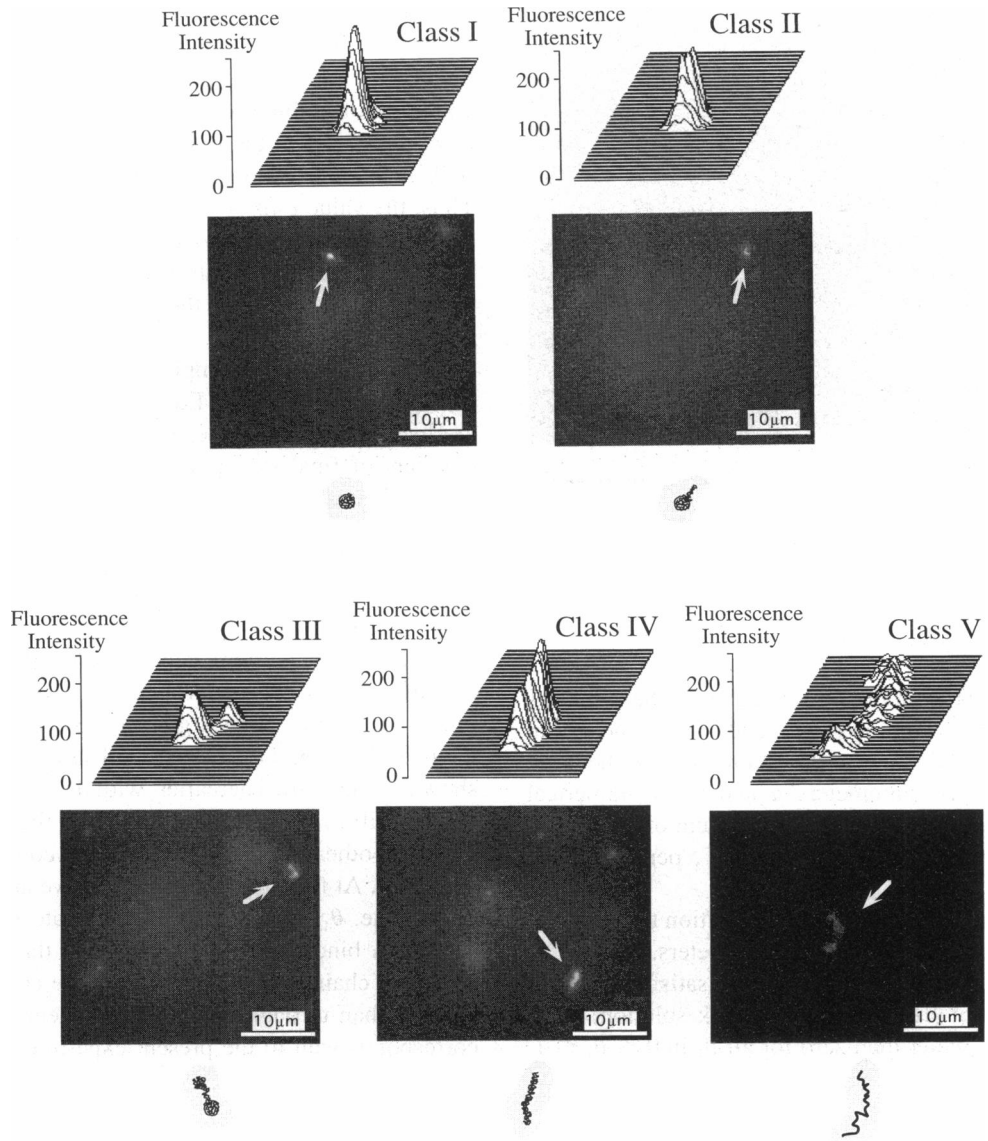

FIGURE 4 Classification of the complex morphology for fluorescent images at [Arg]/[nucleotide] = 0.3. The top panel shows a quasi-3-dimensional profile of the fluorescence intensity of the complex. The middle panel is the actual fluorescence image. The bottom panel is a schematic representation of each fluorescence image together with a depiction of the blurring effect. Class I: tightly compacted complex. Class II: slightly distorted from the class I structure. Class III: Image with several maxima in the fluorescence intensity distribution because of the existence of condensed areas within the complex. Class IV: thick filament with a broad intensity distribution. Class V: random coil state.

species, poly-Arg and Na^+, to the DNA molecule. We regard double-stranded DNA as a uniformly charged, stiff rod-cylinder. We introduce parameters θ_1 and θ_2, representing the degree of binding of cationic species, poly-Arg and sodium ion, to the negatively charged DNA. For simplicity, we do not consider differences in the manner of binding of individual poly-Arg molecules to DNA. Here, we would like to use the term "binding" as the state where the cationic species is located near the negatively charged phosphate groups in DNA. In other words, we consider the binding of cations without distinguishing tight binding and delocalized binding (Oosawa, 1971; Manning, 1978). With this scheme of the binding, the kinetics of the binding of poly-Arg and Na^+ ion can be given as

$$\begin{cases} \dfrac{d\theta_1}{dt} = k_1(c_1 - a\theta_1)(1 - \theta_1 - \theta_2) \\ \dfrac{d\theta_2}{dt} = k_2(c_2 - b\theta_2)(1 - \theta_1 - \theta_2) \end{cases} \tag{1}$$

where the suffixes 1 and 2 correspond to poly-Arg and sodium ion, respectively. θ_i is the variable corresponding to the fraction occupied by the ith chemical species on sites

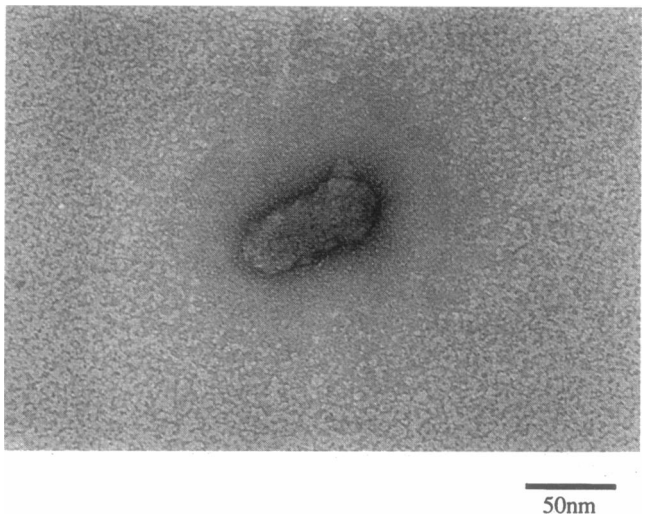

FIGURE 5 Electron microscopic photograph of DNA/p-Arg complex corresponding to a Class I fluorescent image of Fig. 4 at [Arg]/[nucleotide] = 3.0.

with a negative charge along the DNA chain. The symbol k is the rate constant. Parameters c_1 and c_2 are the initial concentrations of poly-Arg and sodium ion in the bulk solution, respectively. Parameters a and b are numerical coefficients representing the saturated amount of binding of the respective species, poly-Arg and Na^+, per one DNA molecular chain.

To draw a phase diagram of Eq. 1 in relation to the actual experiments, we set the following parameters. First, it is noted that the condition $c_1/a \geq 1$ should be satisfied because the concentration of free poly-Arg in bulk solution, $c_1 - a\theta_1$, must not be smaller than zero for all θ_1 in $0 \leq \theta_1 \leq 1$. Similarly, the relationship $c_2/b \geq 1$ is expected in the case of Na^+. Considering that a small amount of free poly-Arg molecules remains in bulk solution at the experimental condition of globule formation, we have adapted the value of $c_1/a = 1.2$. On the other hand, Na^+ ions remain in the bulk under the condition of the excess addition of Na^+. Thus, the value $c_2/b = 1.5$ was chosen. Supposing that the rate of adsorption of poly-Arg is faster than that of Na^+, the ratio of the rate constants was set as $k_2/k_1 = 0.2$. As for the parameters a and b, the ratio were set as $b/a = 1$ for simplicity.

Fig. 6 shows an example of the phase diagram of the time-trajectory based on Eq. 1, indicating that steady states are located along the line, $\theta_1 + \theta_2 = 1$.That is, numerous numbers of final steady states exist along the line of stationary states ($\theta_1 + \theta_2 = 1$), depending on the initial conditions of θ_1, θ_2 ($0 < \theta_1, \theta_2 < 1$ at $t = 0$). Thus, it is concluded that the final, or the stationary, state is not determined solely from the final concentrations of the individual chemicals (DNA, poly-Arg, and Na^+) in the system; the state of the system depends on its past history. For example, let us consider route A. If we consider that the initial addition of poly-Arg corresponds to a change along the line $\theta_2 = 0$ and the system will stop at a certain point as shown in Fig. 6 *a*). Thereafter, with the addition of Na^+, the system will reach state A according to the dynamics of Eq. 1. On the other hand, route B leads the complex into state B (Fig. 6 *b*). At first, the system will move and stop at a point on the line, $\theta_1 = 0$, and then reach state B. Because DNA in state A binds much more poly-Arg than that in state B, the DNA chain is expected to be more compacted through route A than through route B. This theoretical expectation corresponds well to the present experimental findings.

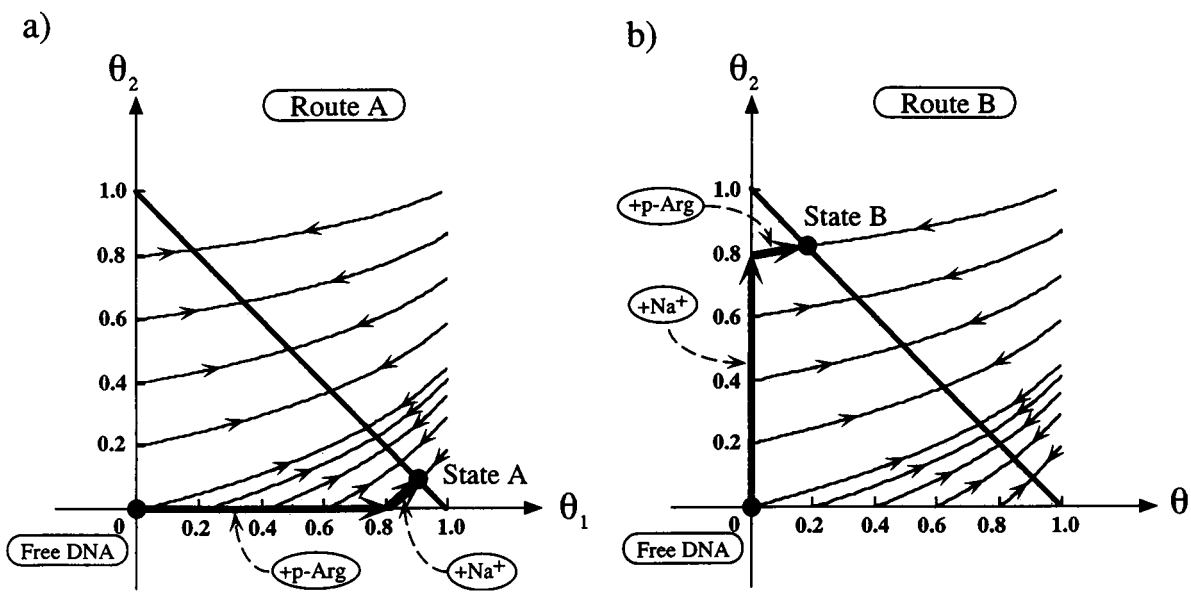

FIGURE 6 Phase diagrams of the trajectory in Eq. 1, indicating that different stationary states are attained depending on the time-procedure, even when the final concentrations of the chemical species in the system are the same.

The most significant point in the above analysis is that the final higher-order structure of single long duplex-DNA chain is not determined solely by the principle of minimum free energy, but highly depends on the time course in the steps of sample preparation. Although we have neglected almost all of the nonlinear effect on the dynamics, including the rather complex interactions between the higher-order structure and the number of the bound ions, the essential aspect on the effect of the time-dependent procedure has been thus interpreted.

As a next step, let us discuss the relationship between the higher-order structure in a DNA chain and the degree of binding with poly-Arg. Even without such a theoretical consideration, it may be easy to expect that enhanced binding of poly-Arg will cause the higher-order structure of DNA to be more compact. However, to gain insight into the effect of the size distribution in a semiquantitative manner, here we would like to develop the following theoretical treatment.

A DNA chain is considered to be a stiff string that contains N segments with persistence length λ. The free energy can be written as (Grosberg and Khokhlov, 1994):

$$\frac{F(\alpha)}{T} = \frac{3}{2}\left(\frac{1}{\alpha^2} + \alpha^2\right) + \frac{BN^{1/2}}{\alpha^3\lambda^3} + \frac{C}{\alpha^6\lambda^6} \tag{2}$$

where the first two terms describe the contribution from the elastic energy, and the third and fourth terms are the contribution from the interaction energy. The variable α is the swelling coefficient, $\alpha = S/S_0$; S is the radius of gyration of DNA chain, whereas S_0 is the gyration radius for a Gaussian chain with the same contour length. With a good approximation, S is expected to be proportional to the long-axis length. B and C are the second and third virial coefficients for the effective interaction among the segments, including solvent effects. In Eq. 2, the effect of counterion binding on free energy has been ignored for simplicity.

Here we suppose that B and C change depending on the degree of binding of poly-Arg, θ_1. We also suppose that B has a negative value because of the bridging effect of poly-Arg between the DNA segments. With these assumptions, the third and fourth terms can be rewritten as

$$\frac{BN^{1/2}}{\lambda^3} \equiv \beta = -m\theta_1 \tag{3}$$

$$\frac{C}{\lambda^6} \equiv \gamma = n\theta_1 \tag{4}$$

where β and γ are the reduced second and third virial coefficients, respectively. We ignored the effect of binding of Na^+ on these coefficients because the effect of Na^+ is expected to be much smaller than that of poly-Arg. Eqs. 3 and 4 show that B becomes more negative and C becomes more positive as the amount of bound poly-Arg increases. For simplicity, we assumed that both B and C are zero when $\theta = 0$ (ideal chain).

The distribution function of the DNA chain is calculated from the following relationship:

$$P(\alpha) = \frac{\alpha^2 \exp(-F(\alpha)/T)}{\int \alpha^2 \exp(-F(\alpha)/T)d\alpha} \tag{5}$$

Fig. 7 *a* exemplifies the theoretical results of the change in the distribution function with $m = 2.0$ and $n = 0.05$. The resulting size distributions deduced from the theory (Fig. 7 *a*) correspond rather well to those in the experiments (Fig. 7 *b*; reconstructed from Fig. 2, route B). We have chosen the values for m and n in the calculation because of the following reason.

It is noted that large m value corresponds to large attractive interaction between DNA segments. It is also to be indicated that, to take into account the effect of self-volume of DNA chain in a proper manner, n should generally be larger/smaller accompanied with the increase/decrease of m. When the parameter m decreases, the bimodal character in the density profile is enhanced. In other words, distinguishable two-peaks exist in the theoretical distribution with small m value. For example at the conditions $m = 0.44$ and $n = 0.001$, clear bimodal distribution is obtained with the above theoretical equation (data are not shown). The distri-

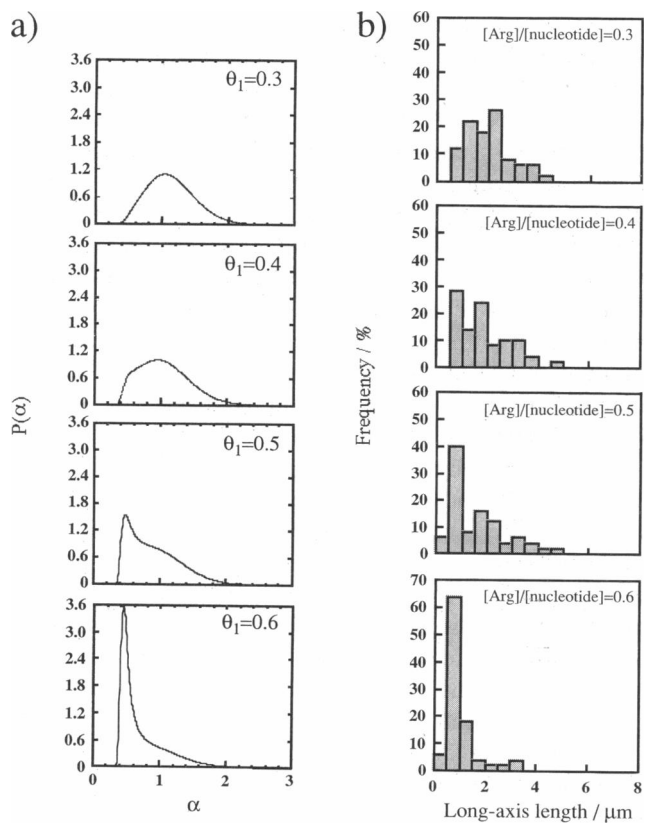

FIGURE 7 (*a*) Changes in the distribution of α (swelling coefficient) depending on the degree of binding of cationic species obtained from Eq. 5, with $m = 2.0$ and $n = 0.05$. (*b*) Changes in the distribution of long-axis length obtained from the experiment with fluorescence microscopy, based on the result for route B in Fig. 2.

bution profile with bimodality corresponds well to the experimental distributions for the coil-globule transition of long DNA induced by small cationic species, such as tricationic amine (Yoshikawa et al., 1996b) and trivalent cobalt complex (Yoshikawa et al., 1996a). It is expected that poly-Arg with a lot of positively charged monomers mediates larger attractive interaction between negatively charged DNA segments than small cationic species do. Thus, in the calculation of Fig. 7 *a*, a large value of m has been chosen to include the large attractive interaction between DNA segments mediated by poly-Arg. As for the parameter n, this should be positive. If this is negative, the globule state becomes infinitely small, which is prohibited in the collapse of actual DNA chain. In addition to this, it is to be mentioned that, when we choose at $n = 0.05$ and $m = 2.0$, the free energy minimum on the side of globule is found to be located around $\alpha = 0.3 \sim 0.5$, corresponding to the experimental trend in the present study.

Thus, these analysis clarify how the amount of bound poly-Arg is related to the conformation of the DNA chain.

CONCLUSION

It has become clear that the higher-order structure of DNA in solution is dependent on the time course of sample preparation. Such a time-dependent effect has been discussed in relation to multistability in the higher-order structure of DNA. The multistability is attributable to the competition between different binding species. Because there exist various species that bind to DNA in the actual environment of living cells, it is expected that the competitive effect, together with the hysteresis effect, contributes significantly to the determination of the higher-order structure of DNA in vivo. In relation to the manner of compaction, or folding, in a DNA chain, it should be noted that DNA chains in vivo must unfold from compacted states to transfer the information in the base sequence into RNA, and then into proteins. In other words, change in the manner of compaction for a DNA chain is expected to be neatly concerned with the efficiency in gene expression. Actually, in the eukaryotic cell nucleus the degree of condensation in chromatin structure is considered to have a close relation to the transcriptional activity (Alberts et al, 1994). It has become clear that, though the active chromatin region with loosely condensed nucleosomal arrangement is often expressed, the heterochromatin region has no transcriptional activity. In this way, the control of transcriptional activity is subject to the degree of condensation by changing the higher order structure of long DNA chain. Thus, the manner of packing DNA chain depending on the past history of its environment is highly expected to be concerned with the mechanism of self-regulation on gene expression.

The present finding that the higher-order structure of long DNA chains reflects their past history suggests another possible informational source in living cells, in addition to the information stored in the primary structure of DNA. Using an analogy to computer architecture, it may be worthwhile to consider that the primary structure of DNA acts as a kind of read-only memory (ROM), whereas the higher-order structure, which reflects the time-course of the interaction with coexisting chemical species, acts as a kind of random-access memory (RAM). Studies on controlling the higher-order structure of DNA by the binding of various chemical species may be important in solving the long-standing problem in life science of how living cells can regulate gene expression using only the one-dimensional information stored as the base sequence along the DNA chain. The textbook explanation is that gene expression is controlled by various kinds of regulatory proteins. However, it should be noted that regulatory proteins are also produced according to the information stored in the primary structure of DNA. Thus, to solve this chicken-or-the-egg problem, other sources of information besides the base sequence should be considered. The new finding in the present study, that the higher-order structure of a collapsed single chain of long DNA can reflect its past history, is expected to shed light on the unsolved problem: How is DNA compaction programmed together with temporal and spatial DNA events inside living cells?

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan. The authors gratefully acknowledge Dr. T. Kanbe for useful advice on the electron microscopic measurement.

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