Real-Time Monitoring of in vitro Transcriptional RNA by Using Fluorescence Correlation Spectroscopy

Yasutomo Nomura*[a, b] and Masataka Kinjo[c]

In vitro transcription is widely used to synthesize small amounts of RNA from recombinant DNA templates.[1] In the conventional analysis of transcripts, northern blotting is used as a technique for size fractioning the RNA in a denaturing gel. In this study, we provide a novel technique to monitor in vitro transcriptional RNA synthesis without any denaturants while the reaction is progressing. Fluorescence correlation spectroscopy (FCS) sensitively measures fluctuations in fluorescence intensity, due to only a few fluorescent molecules that diffuse in and out of a small volume element at the subfemtoliter level in solution, which are dependent on the molecular weight and the concentration.[2–5] Recently, this method was applied to measuring diffusion and the hybridization state of oligo(dT) and poly A in mRNA within living cells.[6] However, it is difficult to detect a specific mRNA by using oligo(dT).

In the present study, two plasmids encoding the luciferase gene and *Xenopus* elongation factor-1α were used as templates for transcription. We determined the interaction between these transcripts and labeled 23-mer oligo-DNAs (GL primer), complementary to the sequence of the luciferase RNA or 30-mer oligo(dA). The fluorescence correlation functions were analyzed by using a simple two-component model with a fast-moving component of free GL primer and a slow-moving component of the hybrid.

The secondary structure of the luciferase RNA (site 1–1700) was simulated. In the predicted structure, three sites (52–54, 57–62, and 68–74) in the 52–74 sequence, complementary to the GL primer, interacted with different sites of the same molecule by base-pairing. This suggests the difficulty of binding between the GL primer and the luciferase RNA after folding to form a secondary structure.

The plasmid-encoding luciferase gene (*pLuc*, site 1–1700) was linearized by *Eco*RV (*pLuc*/*Eco*RV). The site 1–1389 was transcribed from *pLuc*/*Eco*RV. Figure 1 shows typical autocorrelation functions of the fluorescent oligo-DNAs in the reaction solution at the various times after the addition of *pLuc*/*Eco*RV as a template. The normalized autocorrelation function of the GL primer was shifted to the right with a reaction time of 120 min; this suggests that there is an interaction between the probe and the larger molecules. In the case of oligo(dA), the autocorrelation function was not shifted (see insert). The results indicated that the GL primer hybridized with the luciferase transcripts before they folded to form a secondary structure. Furthermore, there was no significant difference between the diffusion time of the GL primer and oligo(dA) in the in vitro transcription system with pXef-1, which is a linearized plasmid DNA that contains the *Xenopus* elongation factor-1α gene (see Table 1). Neither fluorescent oligo-DNA probe was complementary to the pXef-1 transcript. Thus, these results indicated that the GL primer hybridized with transcripts of *pLuc*/*Eco*RV in a sequence-specific manner.

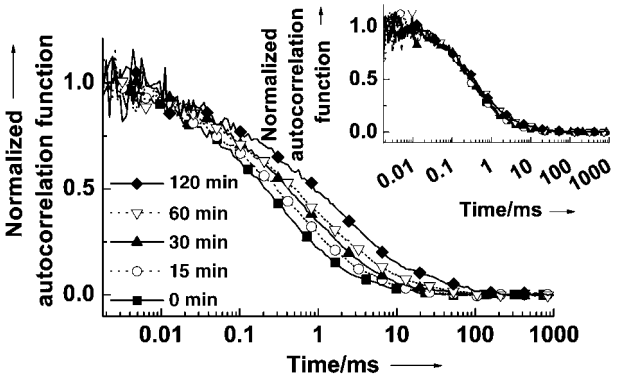

Figure 1. *Normalized autocorrelation functions of in vitro transcription products of* pLuc/Eco*RV. Fluorescence fluctuation was probed with a GL primer and with oligo(dA) (insert). Different curves show autocorrelation functions at various reaction times after the addition of the plasmid. Both symbols and lines represent experimental data. The concentration of* pLuc/Eco*RV was 0.1 μg.*

Although the autocorrelation function of the GL primer just after the addition of *pLuc*/*Eco*RV could be fitted well by using the one-component model, it was difficult to analyze data from 15 to 120 min. In every transcription reaction, the translational diffusion time of the free fast-moving probe was estimated from the autocorrelation function at 0 min, and the mean time was 0.39 ms ($n = 4$) in Table 1. Using this diffusion time of the fast component as a constant value, we analyzed data from 15 to 120 min with the two-component model, and the autocorrelation function could be fitted well. As shown in Figure 2, the fraction of the slow component increased greatly from 0.20 at 15 min to 0.55 at 120 min; this agreed with the relative band intensity from the densitometric analysis after gel electrophoresis. In contrast, the diffusion time was relatively stable, although there was a slight increase from 3.4 to 5.7 ms. The reason for the slight increase is unclear, nevertheless we observed bands near base 1400 at every reaction time in the gel electrophoresis. These data suggested that, using FCS, we detected the increase in number of the slow component rather than that in the length during in vitro transcriptional RNA synthesis.

[a] *Dr. Y. Nomura*
Department of Bio-System Engineering, Faculty of Engineering
Yamagata University, Yonezawa 992-8510 (Japan)
Fax: (+81) 238-26-3357
E-mail: ynomura@yz.yamagata-u.ac.jp

[b] *Dr. Y. Nomura*
Current address:
Department of Environmental Life Science
Graduate School of Medical Science
Yamagata University, Yonezawa, Yamagata 992-8510 (Japan)
Fax: (+81) 238-26-3357

[c] *Dr. M. Kinjo*
Laboratory of Supramolecular Biophysics
Research Institute for Electronic Science, Hokkaido University
Sapporo 060-0812 (Japan)

 DOI: 10.1002/cbic.200400046

Table 1. Diffusion time (ms) of oligonucleotides in transcription system.[a]

Probe	plasmid	none	pXef-1	*pLuc/Eco*RV		*pLuc/Eco*RI	
GL primer	fitting	1 component	1 component	2 components		2 components	
				Fast	Slow	Fast	Slow
	Diffusion time	0.38	0.58[d]	0.39	5.71	0.35	1.66
		(0.05)	(0.15)	(0.03)	(0.17)	(0.05)	(0.32)
	Fraction	–	–	0.45	0.55	0.49	0.51
				(0.10)	(0.10)	(0.15)	(0.15)
	Sample[b]	7	2	4		3	
	Data points[c]	26	7	19		12	
Oligo (dA)	fitting	1 component	1 component	1 component		1 component	
	Diffusion time	0.33	0.61[e]	0.64[f]		0.63[f]	
		(0.04)	(0.27)	(0.14)		(0.09)	
	Sample	2	3	3		3	
	Data points	9	11	9		11	

[a] Each diffusion time is the mean (SD) at 120 min after the addition of plasmid. [b] The values were measured in the different numbers of samples shown in the table. [c] Three to five values were obtained from each sample. [d] Significantly different from values of GL primer in the absence of pXef-1, $P < 0.005$. [e] Significantly different from values of oligo A in the absence of pXef-1, $P < 0.01$. [f] Significantly different from values of oligo A in the absence of the plasmids, $P < 0.005$.

in Figure 3. FCS measurement was carried out at 120 min after the addition of plasmids. For the reaction from 5 to 10 ng of plasmid DNA, only a small fraction of the slow component was observed between 0.02 and 0.03. At 100 ng of *pLuc/Eco*RV plasmid DNA, the fraction increased to 0.53. The transcription reaction reached the plateau phase at 400 ng. According to the instruction manual of this transcription system, 20 μL reaction mixture containing 1 μg of templates that encode for 1.9 kb transcript synthesized 50 μg of RNA after 120 min of incubation. Although this suggests that the contents

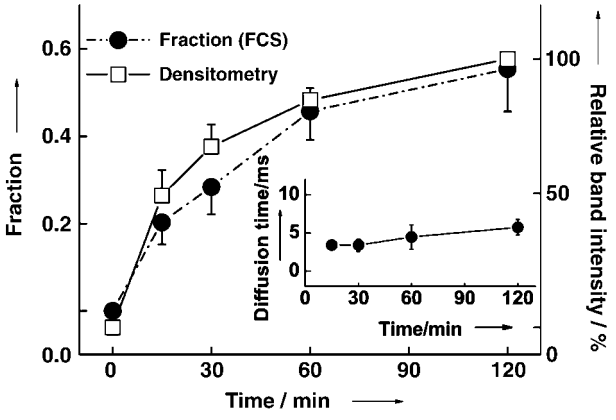

Figure 2. *Fractions and diffusion times (insert) of the slow component in* pLuc/EcoRV *transcripts at different reaction times (*n = *4). A GL primer was used. Each autocorrelation function was fitted to the two-component model. The diffusion time of the fast component was fixed at 0.39 ms. The band intensities of transcripts from gel electrophoresis were also estimated.*

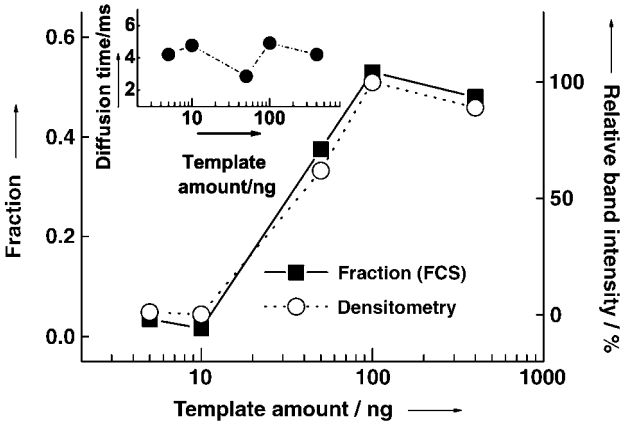

Figure 3. *Dependency of template amount on fraction and diffusion time (insert) of the slow component in the transcription reaction with* pLuc/EcoRV *at 120 min. A GL primer was used. The band intensities of transcripts from gel electrophoresis were also estimated.*

Next, *pLuc* was linearized by *Eco*RI (*pLuc/Eco*RI). The site 1–638 was transcribed from *pLuc/Eco*RI. Using the GL primer, we compared the diffusion times of transcripts of *pLuc/Eco*RV with those of *pLuc/Eco*RI (Table 1). The fraction of the slow component that had a diffusion time of 5.71 ms in the case of *pLuc/Eco*RV increased with reaction time and was 0.55 at 120 min. On the other hand, in *pLuc/Eco*RI, the fraction of the slow component increased to 0.51 at 120 min, but the diffusion time was 1.66 ms. Each transcript was also analyzed by gel electrophoresis after being quantified by FCS measurement. As expected, bands near bases 600, 1400, and 1900 were detected for transcripts with *pLuc/Eco*RI, *pLuc/Eco*RV, and pXef-1, respectively (data not shown). Therefore, FCS can measure diffusion time dependent on base length.

The autocorrelation functions of fluorescent probes in reaction solutions that contained from 5 to 400 ng plasmid DNA were analyzed by using the two-component model, as shown of our transcripts would be much more than that of the labeled primer, the reason for the observation of a large fraction of unbound probes is unclear. In contrast to the changes in this fraction, the diffusion time varied between 2.9 and 4.9 ms, but the upward tendency were not observed. These results agree with those from densitometric analysis of the gel.

Recently fluorescence resonance energy transfer (FRET) has been used to detect the specific mRNA in transcription systems in in vitro and in living cells.[7,8] Since FRET, unlike FCS, depends on the real distance between donor and acceptor, the sequences of the probes must be based on the 3D structure of RNA. Northern analysis is the only conventional method for size fractionating RNA, but this must be performed on the denatured gel. In contrast, FCS did not require any denaturants because the hybrid of the probe with the synthesized RNA before the formation of secondary structure was measured directly. Furthermore, since FCS measurement

causes little damage, we can use the sample even after measuring for different experiments.

FCS permits real-time monitoring of specific RNA in an in vitro transcription system in which the fluorescent oligonucleotide probe was complementary to the coding sequence of RNA.

Experimental Section

The in vitro transcription reaction was carried out with a T7 MEGAscript (Ambion) kit system. The two expression plasmids used in this study were *pLuc* (Promega) and pXef-1(Ambion). Two fluorescent oligo-DNAs were purchased from Sigma Genosys. One was 5′-rhodamine green-CTTTATGTTTTTGGCGTCTTCCA-3′ (23-mer). The other was 5′-rhodamine green-oligo(dA)-3′ (30-mer).

The sequence of luciferase RNA used for the simulation was obtained from the Promega Corp. Simulation of the secondary structure of the luciferase RNA was performed by using Vienna RNA software.[9] FCS measurement was performed by using a ConfoCor fluorescence correlation measurement system (Carl Zeiss Jena GmbH, Jena, Germany) as described elsewhere.[10] The in vitro transcription solution was separated by electrophoresis on formaldehyde denaturing gel (agarose 1%).

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research (C) (2) (14580761) from the Ministry of Education, Science, Sports and Culture of Japan.

Keywords: correlation · diffusion · fluorescence spectroscopy · hybridization · RNA

[1] R. Davis, *Curr. Opin. Biotechnol.* **1995**, *6*, 213 – 217.
[2] S. Maiti, U. Haupts, W. W. Webb, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 11753 – 11757.
[3] R. Pecora, S. Aragon, *J. Chem. Phys.* **1976**, *64*, 1791 – 1803.
[4] N. G. Walter, P. Schwille, M. Eigen, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 12805 – 12810.
[5] M. Kinjo, R. Rigler, *Nucleic Acids Res.* **1995**, *23*, 1795 – 1799.
[6] J. C. Politz, E. S. Browne, D. E. Wolf, T. Pederson, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 6043 – 6048.
[7] Y. Sei-Iida, H. Koshimoto, S. Kondo, A. Tsuji, *Nucleic Acids Res.* **2000**, *28*, E59.
[8] A. Tsuji, H. Koshimoto, Y. Sato, M. Hirano, Y. Sei-Iida, S. Kondo, K. Ishibashi, *Biophys. J.* **2000**, *78*, 3260 – 3274.
[9] I. L. Hofacker, *Nucleic Acids Res.* **2003**, *31*, 3429 – 3431.
[10] Y. Nomura, H. Tanaka, L. Poellinger, F. Higashino, M. Kinjo, *Cytometry* **2001**, *44*, 1 – 6.

Received: February 13, 2004
Early View Article
Published online on November 3, 2004

