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Ultrasensitive hybridization analysis using fluorescence correlation spectroscopy

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ABSTRACT

The hybridization of fluorescently tagged 18mer deoxyribonucleotides with complementary DNA templates was analysed by fluorescence correlation spectroscopy (FCS) in a droplet under an epi-illuminated fluorescence microscope at the level of single molecules. The interaction can be monitored by the change in the translational diffusion time of the smaller (18mer) primer when binding to the bigger (7.5 kb) DNA containing the complementary sequence. The hybridization process in the presence of template M13mp18 ssDNA was monitored in a small volume (2×10^{-16} l) at various temperatures. The Arrhenius plot of the association rate constant shows that the activation energy was 38.8 kcal/mol, but the hybridization process may involve several components. The titration experiment suggested that ~2 primers can be associated with one template DNA at 40°C. Results of a simple homology search for the sequences complementary to the primer indicate the existence of additional sites of lower specificity.

INTRODUCTION

Hybridization is widely used for gene detection and base sequencing and supports our understanding not only of the structure, organization and expression of genes (1–3), but also of higher order structures such as tRNA (4). Quantification of the hybridization process can be measured in a variety of ways. In this paper we present a new and extremely sensitive way to monitor the hybridization process by using fluorescence correlation spectroscopy (FCS). In FCS, thermal fluctuations of molecules excited to fluorescence are observed and correlated. When excitation and observation are carried out in extremely small confocally defined volume elements (0.2 fl), fluctuations at the level of single molecules can be observed (5–9). FCS can be applied to extremely dilute solutions (10^{-8}–10^{-15} M) and the sensitivity of detection is so high that single molecules can be detected (8,10). In particular, the interaction of a fluorescent ligand with a larger target can be measured by the correlation function describing the diffusion of bound and free ligand (6) without the necessity of separation, e.g. on a filter or by column chromatography. We demonstrate the efficiency of FCS analysis in the case of the interaction between an 18 base primer and M13 DNA as one example at nanomolar concentrations in order to obtain the stoichiometry, kinetics and thermodynamics of the interaction.

Analysis shows that the method presented can be applied to analysis of the interaction of a specific primer with a target DNA or RNA sequence in solution and will also be able to give an insight into the tertiary structure of the DNA (RNA) sequence from the accessibility of nucleotide bases for interaction with the primer.

In the present paper we report the measurement of the hybridization rate between M13 DNA and a universal sequencing primer in a volume element of ~0.24 fl.

MATERIALS AND METHODS

Bodipy-labelled and tetramethylrhodamine (TMR)-labelled sequencing primers (5′-TGTAAAACGACGGCCAGT-3′, Fluoro Tide M13/pUC(–21) primer, 5′-Bodipy 558/568) was purchased from Molecular Probes Inc. M13mp18(+) strand DNA (7250 bases) was from Pharmacia.

The experimental set-up and theoretical background of FCS have been described previously (5–9) (Fig. 1). The set-up consists of an argon laser (Spectra Physics Model 165), an epi-illuminated microscope with a water emersion objective (Zeiss Neofluar, 63 × 1.2), an avalanche photodiode (EG&G, SPCM-100 PQ) in photocounting mode as detector and a digital signal correlator (ALV Laser Vertriebsgesellschaft GmbH, ALV-5000).

The data analysis was performed using non-linear least squares parameterization for calculating the normalized mean square deviation between data and model (11).

The ligand–target interaction can be analysed by measuring the change in diffusion time by FCS (6) for the case that diffusion time is very much smaller than chemical relaxation time. The average number of fluorescent molecules per volume element (n) and translational diffusion constant of free primer (D_{free}) and associated primer (D_{bound}) were fitted to the autocorrelation function [$G(t)$] describing the diffusion in a three-dimensional Gaussian volume element (6):

$$G(t) = 1 + 1/n\langle\{1 - y/[(1 + t/\tau_{free})(1 + s^2 t/\tau_{free})^{1/2}]\} + \{y/[(1 + t/\tau_{bound})(1 + s^2 t/\tau_{bound})^{1/2}]\}\rangle$$

where y = fraction bound primer, $\tau_{free} = w_0^2/4D_{primer}$, $\tau_{bound} = w_0^2/4D_{DNA}$ and $s = w_0/z_0$, where w_0 is the radius of the volume

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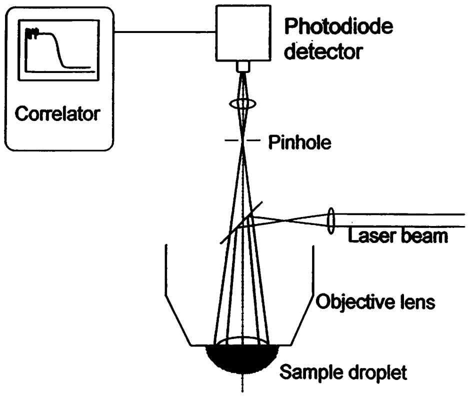

Figure 1. Schematic diagram of the fluorescence correlation spectroscopy (FCS) set-up.

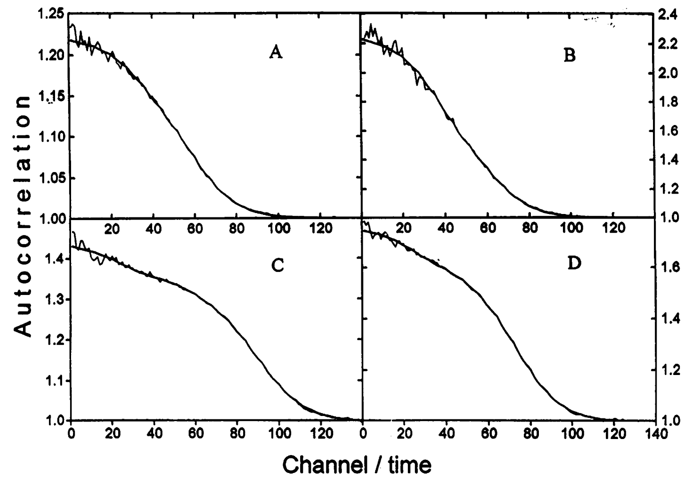

Figure 2. Autocorrelation function of (**A**) Bodipy-labelled primer, τ_{free} = 0.166 ms, n = 4.5, (**B**) tetramethylrhodamine-labelled primer, τ_{free} = 0.181 ms, n = 0.864, (**C**) Bodipy-labelled primer–M13DNA complex after 256 min incubation at 40°C, τ_{bound} = 2.943 ms, τ_{free} = 0.166, y = 0.964 and (**D**) tetramethylrhodamine-labelled primer–M13DNA complex after 5 min incubation at 57°C, τ_{bound} = 3.6 ms, τ_{free} = 0.18 ms, y = 0.908.

element and $2z_0$ is its length. The fractions of free and hybridized primer are defined by the value of y.

The fluorescence of the 5′-Bodipy-labelled and TMR-labelled primers was excited at 514.5 nm and the emission was measured after passing through a KV550 cut-off filter (Schott, Germany).

We carried out two protocols for analysis of hybridization with a total amount of 1 pmol primer and template, one applied to the Bodipy-labelled primer and the other to the TMR-labelled primer. For kinetic experiments with the Bodipy-labelled primer, 50 nM primer and 50 nM template DNA were prepared in 0.18 M NaCl (2). The sample solution (20 µl) was incubated at 40°C. The FCS measurement was carried out at 20°C. After one measurement the sample solution was collected from the surface of the lens and the incubation procedure was continued. In order to avoid evaporation of the droplet, a small chamber was mounted around the droplet hanging on the surface of the lens. The same sample was used for the whole experiment. For kinetic experiments with the TMR-labelled primer the incubation procedure was carried out at 30, 37 and 57°C. In order to terminate the reaction after each incubation interval, an aliquot volume (1 µl) of reaction mixture was transferred quickly to ice-cold 0.18 M NaCl solution (20 µl) and measured at room temperature.

For titration experiments the concentration of template DNA was fixed at 50 nM and that of primer was changed. The incubation was carried out at 40°C for 128 min. The FCS measurement was at 20°C.

RESULTS

In order to excite fluorescence of labelled molecules in a volume element of 0.2 fl, an intensity of 250 kW/cm^2 at 514.5 nm was used. The average counting rate for rhodamine 6G, Bodipy-labelled primer and TMR-labelled primer under these condition are 100 000, 18 500 and 19 400 counts/s/molecule, respectively. The size of the volume element was calibrated by the diffusion of rhodamine 6G. A diffusion time of 0.04 ms for rhodamine 6G was obtained, corresponding to a volume element with dimensions w_0 = 0.2 µm and z_0 = 1.0 µm. The translational diffusion time for the Bodipy-labelled and the TMR-labelled primers was found to be 0.166 and 0.181 ms, respectively (Fig. 2A and B).

The formation of a complex between primer and template was well identified from the change in autocorrelation function. The translational diffusion time of the Bodipy-labelled and the TMR-labelled primer–DNA complexes were 2.94 and 2.86 ms, respectively (Fig. 2C and D). Due to different translational diffusion times between the free primer (18 bases) and the bound primer (primer–template complex, 7.5 kb), we were able to quantify the fraction of the primer hybridized. The diffusion times of free and bound primers were determined independently, for example 0.166 ms for free and 2.943 ms for bound in the case of the Bodipy-labelled primer. Although the unbound and bound primers were not separated after incubation, it was possible to measure the fraction of hybridization. The fraction of hybridized primer was plotted versus time (Fig. 3). The evaluation was performed according to $y(t) = 1 - [1/(1 + kC_0t]$, where k = rate constant for association and C_0 = initial concentration of single-strand primer and single-strand template DNA respectively. The activation energy was also calculated from the temperature dependence of k (insert in Fig. 3). The high sensitivity of detection is demonstrated by the fact that the fluorescence signals were collected on average from 1–4 labelled primer molecules per volume element (n, Fig. 2).

A titration experiment was carried out in order to define how many binding sites are available for this primer in M13 DNA. Figure 4 shows that the hybridization was saturated at a primer:template ratio of 2:1. This indicates that two primers may bind to one M13 template DNA and suggests the presence of an additional site with similar affinity under the conditions applied (hybridization at 40°C, FCS measurement at 20°C). Therefore, a homology search was carried out in the M13mp18 DNA strand for a sequence complementary to the primers using Seqaid II v.3.81 (12). Possible sites proposed in M13mp18 DNA for this primer are shown in Figure 5.

DISCUSSION

Kinetics

We demonstrate a new method for the analysis of the association rate of DNA in tiny volume elements (0.24 fl). The experimental results suggest an association rate constant of 3.5 × 10^4/M/s at

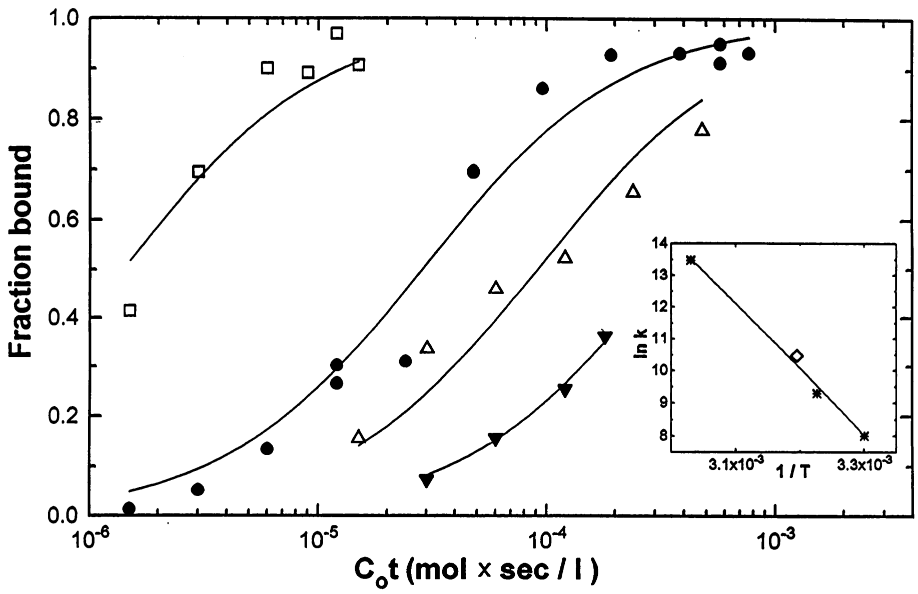

Figure 3. Time course of hybridization between universal sequencing primer (50 nM) and ssM13 DNA (50 nM) in 0.18 M NaCl. The reaction was carried out at 40°C (●) with the Bodipy-labelled primer and at 57 (□), 37 (Δ) and 30°C (▼) with the tetramethylrhodamine-labelled primer. A solid line is the second order fitting (—) versus time using $1 - [1/(k * C_0t + 1)]$, where k = rate constant for association, C_0 = initial concentration of single-strand primer and single-strand template DNA, respectively. The association rate constants at 30, 37, 40 and 57°C were 3×10^3, 11×10^3, 35×10^3 and 716×10^3/M/s, respectively. The activation energy (38.8 kcal/mol) was calculated from an Arrhenius plot (insert) for the tetramethylrhodamine-labelled (*) and the Bodipy-labelled (◇) primers.

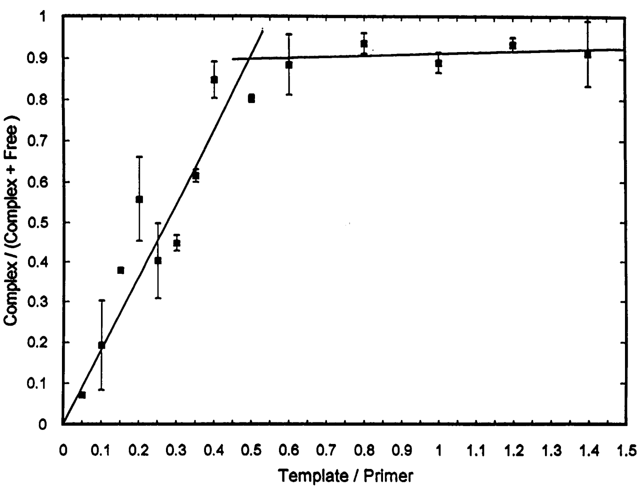

Figure 4. Effect of DNA:primer ratio on hybridization. Hybridization using 50 nM primer was carried out in 0.18 M NaCl at 40°C for 128 min. The concentration of M13mp18 DNA was changed in the range 2.5–70 nM. Data of four experiments were combined (average ± SD, vertical line) and fitted to a first order rate equation by non-linear least squares fitting.

40°C. This value is comparable with the rate constant obtained for an 18mer nucleotide of 8.1×10^4/M/s and of 0.6×10^4/M/s after correction for 0.18 M NaCl (13–15). A value of 2.3×10^5/M/s is reported for a 20mer under PCR conditions (14).

Moreover, the temperature dependence of the rate constant shows a linear relationship (Fig. 3 insert) and identical behaviour for Bodipy and TMR. Wetmur reported biotinylated probes to be slightly reduced in T_m, but the labelling did not affect the rate constant (14). Our results may indicate that both these fluorescent probes do not affect the rate constant of hybridization.

The activation energy was reported to be from –4 to –9 kcal/mol for the association of oligoA:oligoU and that of an oligomer having GC pairs to be, for example, 13 kcal/mol for $A_4G_2 + C_2U_4$ and 4.5 kcal/mol for $G_3C + GC_3$ (16). The Arrhenius plot of the rate constant shows an activation energy for the primer template association of 38.8 kcal/mol (Fig. 3 insert). The large positive activation energy in this experiment seems to be needed for unfolding the long template DNA (7.5 kb) before association with the primer.

Stoichiometry

From the results of the homology search, various sites with different degrees of similarity to the authentic primer site become evident. These sites contain mismatches (nos 2 and 9–11 in Fig. 5) or loop formations (nos 3, 4, 6–8 and 12–14 in Fig. 5). In earlier studies of the thermodynamics of d(CGCGAATTCGCG) one mismatch nucleotide decreases the T_m by ~17°C from normal (17). Also, the occurrence of single-strand loop regions influences the free energy of interaction (18). On the other hand, recent work reported new thermodynamic properties for mismatches [G:A (19), G:U (20)] and for dangling thymidine residues (21). Moreover, methylphosphonate linkages make stable conformations in low salt (0.01 M) conditions (22).

Compared with the authentic primer binding site, hybridization with these sites, however, should be much less stable at 40°C. At 20°C, the temperature at which the FCS analysis was carried out for technical reasons, increased stability and primer binding can be expected and is likely the reason for the additional binding site observed. The occurrence of these extra sites (Fig. 5, 2–14) would be a reason for double sequence patterns, which are known as

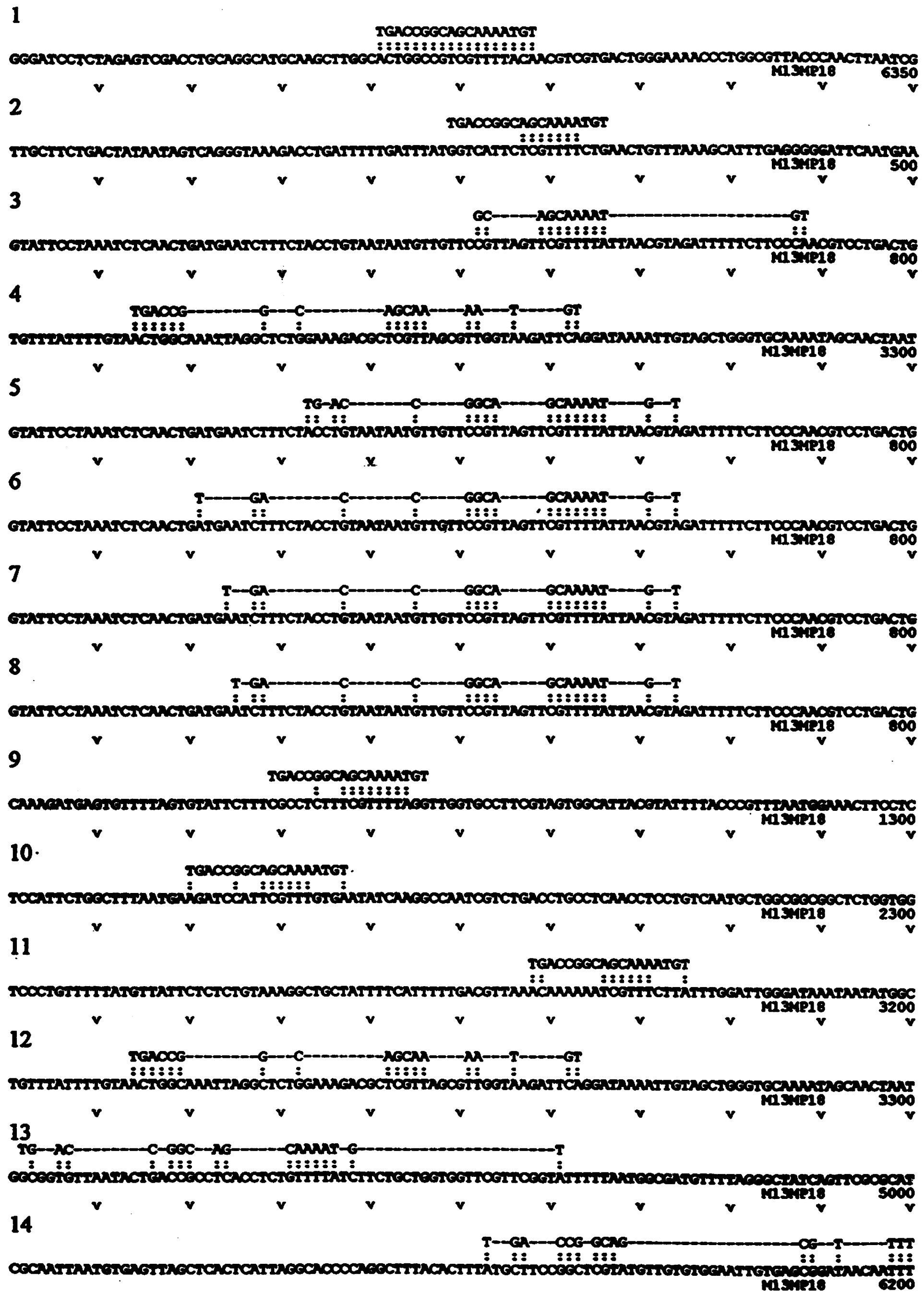

Figure 5. Sites proposed for primer association in M13mp18 DNA. 1, full associating site; 2–14, partial associating sites. The positions are numbered from base 1 on the right side. V is a marker for each 10 nt.

'extra' or 'ghost' bands due to second site primer annealing in the usual sequencing procedure at 37°C (23).

It is evident from the present results that for a quantitative analysis of base-specific hybridization a careful assessment of the temperature of hybridization and analysis is important. Given these precautions, FCS provides a handy and very sensitive tool for analysing conditions which may enable one to design not only more efficient primers for sequencing, PCR and hybridization, but also more efficient antisense and antiviral nucleotide sequences.

Fluorescence correlation analysis

Compared with the standard label in FCS (rhodamine 6G), both Bodipy and TMR have a lower emission rate/molecule. A detailed analysis (not shown) indicates an increased population in the triplet state, as well as an increased triplet relaxation time (24) (2–20 μs) under standard excitation condition (250 kW/cm^2 at 514.5 nm) as a likely reason for the reduced emission intensity. The increased formation of triplet states in benzopyrenes due to adduct formation with nucleic acids has been reported previously (25).

The separation of free and DNA-bound primer is based on their diffusion times, which differ by a factor of 10. The fraction of bound primer y is directly available from the autocorrelation function, making any physical separation unnecessary. Due to the high sensitivity and small volumes used, very small amounts of material (fmol) are needed. Since FCS can be used to detect single molecules, we foresee that this method will be able to identify specific RNA and DNA sequences in solution, as well as in the cellular environment.

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