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Sigma cycle during *in vitro* transcription: Demonstration by nanosecond fluorescence depolarization spectroscopy

(RNA polymerase/gene transcription/fluorescence polarization/Brownian motion/macromolecular association-dissociation)

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ABSTRACT **Studies of RNA chain initiation have suggested that the σ subunit of *Escherichia coli* RNA polymerase (RNA nucleotidyltransferase; nucleosidetriphosphate: RNA nucleotidyltransferase; EC 2.7.7.6) is released from the enzyme–template complex during transcription and may be reused by another core polymerase. Nanosecond fluorescence depolarization spectroscopy was used to follow the σ cycle. Isolated σ subunit labeled with the fluorescent probe dansyl (DNS) chloride bound stoichiometrically to core polymerase and stimulated transcription of phage T7 DNA to the same extent as did unlabeled σ. DNS-σ showed an exponential fluorescence anisotropy decay corresponding to a rotational correlation time of about 100 nsec. This value was unaffected by addition of T7 DNA, but increased about 6-fold when core polymerase was added, and increased further when T7 DNA was added. Such increases are expected for the formation of molecular complexes. Using the anisotropy decays for free DNS-σ and DNS-σ–core enzyme bound to T7 DNA, we calculated theoretical decay curves for various mixtures of free and bound σ. Comparison of the observed anisotropy decay with the calculated curves indicated that about 55% of DNS-σ was released from the enzyme–T7 DNA complex in the presence of four nucleoside triphosphates under low salt conditions. Sigma release did not occur if rifampicin was added prior to addition of four nucleoside triphosphates or if only three nucleoside triphosphates were present. After σ was released, addition of core polymerase with rifampicin reduced the free σ to less than 15%, indicating that the released σ was accessible to the added core enzyme. Thus these studies have provided physical evidence for the σ cycle during *in vitro* transcription.**

The transcription of a bacterial genome is primarily mediated by a single RNA polymerase (RNA nucleotidyltransferase; nucleosidetriphosphate:RNA nucleotidyltransferase; EC 2.7.7.6). The purified *Escherichia coli* RNA polymerase holoenzyme has a subunit structure of $\alpha_2\beta\beta'\sigma$, with molecular weights for the subunits of 40,000, 155,000, 165,000, and 90,000, respectively (1, 2). The holoenzyme can be reversibly dissociated to yield the core enzyme ($\alpha_2\beta\beta'$) and the σ subunit. Like the holoenzyme, core polymerase is catalytically active, but differs in transcribing native double-stranded DNA nonspecifically and inefficiently (3, 4).

The σ subunit by itself has no catalytic function. Its role in gene transcription is 2-fold. (*a*) The σ specificity: σ promotes specific initiation of RNA chains that yields asymmetric transcription resembling *in vivo* RNA products (1). (*b*) The σ cycle: σ stimulates RNA synthesis by increasing the rate of initiation through its catalytic reuse by core polymerase (5).

Abbreviations: DNS, ansyl group or 5-dimethylaminonaphthalene-1-sulfonyl group; DNS-σ, dansylated σ subunit; NTP, nucleoside triphosphate.

Based on their *in vitro* transcriptional studies, Travers and Burgess (5) first proposed the following σ cycle: σ initially forms a complex with core polymerase, which is able to bind to promotor sites on DNA and to initiate specific RNA synthesis. During or after initiation, σ is released from the enzyme–DNA complex and may then be reused by another core polymerase molecule to initiate a new RNA chain.

Evidence for the physical separation of σ from the enzyme–DNA complex after RNA chain initiation has been provided by use of polyacrylamide gel electrophoresis for *Azotobacter* and *E. coli* RNA polymerases (6, 7), or by use of sucrose gradient centrifugation for *Pseudomonas* RNA polymerase (8). However, the evidence is not conclusive because electrical or centrifugal force used could influence the interaction between σ and core polymerase. Even if the effect of the external forces is negligible, these studies only indicate a weakening of the σ–core polymerase interaction during transcription, resulting in the subsequent separation of σ from core polymerase by the electric field or centrifugal force, and do not prove the physical release of σ due to RNA synthesis.

This communication presents a direct demonstration by nanosecond fluorescence depolarization spectroscopy of the physical release of σ during transcription. This technique measures the rate of molecular rotation (Brownian rotational diffusion) and thereby provides information concerning the sizes, shapes, and aggregation states of macromolecules (9, 10)

MATERIALS AND METHODS

RNA polymerase holoenzyme was purified from cells of *E. coli* B as described previously (11). The purity of the enzyme was >95% as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The σ subunit and core polymerase were isolated from the holoenzyme by the procedures of Berg *et al.* (12). Phage T7 DNA was prepared as described by Sadowski (13). Phage fd DNA was a gift from Dr. J. Hurwitz. Dansyl chloride (DNS-Cl) (Sigma) adsorbed on Celite was prepared by the method of Rinderknecht (14). Determinations of RNA polymerase activity and measurements of RNA chain initiation using [γ-^{32}P]ATP were performed as described in a previous paper (15).

To label the σ subunit, the free sulfhydryl groups in σ were first blocked with potassium tetrathionate (15). A 50- to 100-fold molar excess of [^{3}H]dansyl chloride (14 cpm/pmol) adsorbed on Celite was then added to the SH-blocked σ [1–2 mg in 0.02 M K_2HPO_4, 0.05 M KCl, 10^{-4} M EDTA, 5% (v/v) glycerol, pH adjusted to about 10 with 1 M

Na_2CO_3 immediately prior to the reaction]. The reaction mixture was incubated for 5–10 min at 3° with gentle stirring, passed through a Sephadex G-25 column to remove free dansyl chloride, and centrifuged in a SW 50.1 rotor for 22 hr at 49,000 rpm on a 15–30% glycerol gradient. Fractions sedimenting in the region of unmodified σ were pooled and stored at 3°. Immediately before use, the labeled σ was treated with 10^{-2} M dithiothreitol to regenerate free sulfhydryl groups and dialyzed against Buffer A (0.05 M Tris, pH 7.8, 0.05 M KCl. 0.01 M $MgCl_2$, 10^{-4} M dithiothreitol, and 10^{-4} M EDTA). Under these labeling conditions, dansyl chloride presumably reacted with the ϵ-amino groups of lysine residues on the surface of the protein (16). The stoichiometry of labeling was 1 to 5 DNS per σ molecule as determined by [^{3}H]DNS incorporation.

Fluorescence excitation and emission spectra were recorded on a Hitachi Perkin-Elmer fluorescence spectrophotometer (model MPF-3) equipped with a corrected spectra accessory. Excited-state lifetime and time-dependent emission anisotropy measurements were made by the single photon counting technique using an Ortec 9200 nanosecond fluorescence spectrometer. The light source was an air-filled spark-gap lamp operating at 20–40 kHz, which gave light pulses with a full width at half-maximum of approximately 1 nsec. The exciting light was passed through a Corning CS 7-60 filter and polarized in the y direction by a Polaroid sheet polarizer. The emitted light was detected along the z direction through a Kodak Wratten 2-A filter. A rotating polarizer on the emission side was used to select the x and y components of the fluorescence, $F_x(t)$ and $F_y(t)$. The time-dependence of the total emission, $S(t) = F_y(t) + 2F_x(t)$, was obtained by setting the emission polarizer at 54.7° to the y direction. The fluorescence intensity as a function of time was recorded on a multichannel analyzer interfaced to a PDP-11 digital computer. The method of moments (17) was used to deconvolute and analyze $S(t)$ in terms of single or double-exponential decays

$$S(t) = \Sigma a_i e^{-t/\tau_i} \quad [1]$$

where a is the amplitude factor and τ is the excited-state life-time. The time-dependent emission anisotropy, $A(t)$, defined as (18, 19)

$$A(t) = \frac{F_y(t) - F_x(t)}{S(t)} \quad [2]$$

was calculated by the computer.

If the macromolecule is a rigid sphere, $A(t)$ decays exponentially according to the relation:

$$A(t) = A_0 e^{-t/\phi} \quad [3]$$

where A_0 is the emission anisotropy in the absence of molecular motion and ϕ is the rotational correlation time. The rotational correlation time is related to the volume v of the hydrated sphere, the viscosity η of the solution, the absolute temperature T and the Boltzman constant k by

$$\phi = \frac{v\eta}{kT} \quad [4]$$

If the macromolecule is not a rigid sphere, $A(t)$ shows a multiexponential decay (20, 21). However, most proteins studied thus far by nanosecond polarization techniques exhibit a single rotational correlation time sufficiently large to correspond to the motion of the rigid spherical particle (10). This was also the case in the present studies. Thus the value of ϕ was obtained by a linear least squares analysis of semilogarithmic plots of $A(t)$.

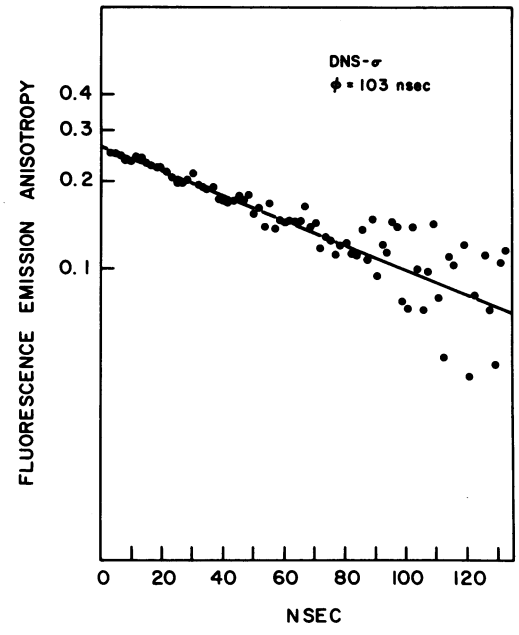


FIG. 1. Time-dependent emission anisotropy of DNS-σ. The solid line is a theoretical plot for ϕ = 103 nsec and A_0 = 0.26. The sample contained 35 pmol of DNS-σ in 0.5 ml of Buffer A. The temperature was maintained at 23°.

In principle, $F_y(t)$ and $F_x(t)$ must be deconvoluted to yield $A(t)$. However, deconvolution was not necessary in this study since both τ and ϕ are sufficiently long compared to the exciting light-pulse duration and the instrument response time.

RESULTS

The fluorescent-labeled σ (DNS-σ) was as active as unlabeled σ (>80%) in stimulating the transcription of T7 DNA by core polymerase. The fluorescence excitation and emission maxima of DNS-σ were at 340 nm and 520 nm, respectively. The nanosecond emission kinetics of DNS-σ could be fitted by two excited-state lifetimes, τ_1 = 4 nsec and τ_2 = 20 nsec, with relative amplitudes, a_1 = 0.4 and a_2 = 0.1. Neither the fluorescence spectra nor the excited-state lifetimes of DNS-σ were altered by addition of core polymerase.

The time-dependent fluorescence emission anisotropy of DNS-σ is shown in Fig. 1. To a good approximation the plot of ln $A(t)$ versus time is a straight line, the slope of which yields a rotational correlation time of 103 nsec. The observations that DNS-σ shows two different excited-state lifetimes and yet only a single rotational correlation time can be understood from theoretical considerations (20) of anisotropy decay in the case of randomly labeled spherical macromolecules. These considerations reveal that $A(t)$ is independent of $S(t)$. The value of ϕ for DNS-σ is not affected by addition of T7 DNA, supporting the previous report (22) that isolated σ does not bind to DNA. When a stoichiometric amount of core polymerase was added, the rotational correlation time increased to about 600 nsec. (Fig. 2). If an excess of T7 DNA was then added, the value of ϕ increased further to 800 nsec (data not shown). Such changes are expected, since the formation of larger molecular complex will slow down the rate of molecular rotation and hence increase the value of ϕ.

When the DNS-σ–core polymerase–T7 DNA complex was

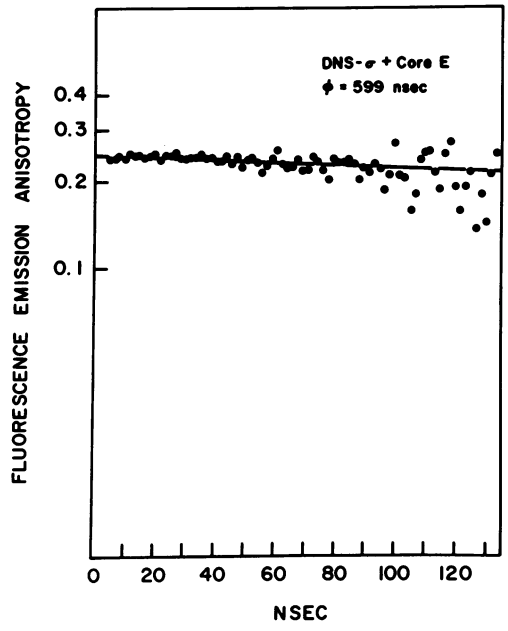


FIG. 2. Time-dependent emission anisotropy of the DNS-σ-core polymerase complex. The solid line is a theoretical plot for ϕ = 599 nsec and A_0 = 0.25. The sample and conditions were the same as in Fig. 1 except that 35 pmol of core polymerase was added and incubated for 5 min prior to data collection.

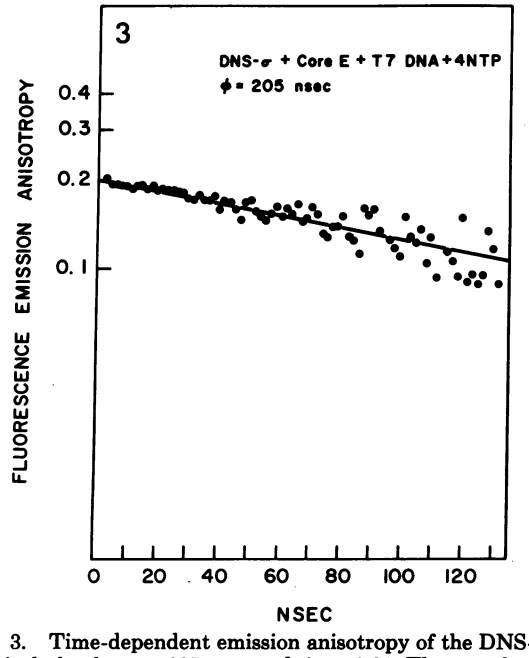
incubated with four nucleoside triphosphates to start RNA synthesis under low salt conditions in which there was no significant reinitiation of RNA chains, the rotational correlation time decreased from 800 nsec to 205 nsec as shown in Fig. 3. If all DNS-σ had been released from the enzyme–DNA complex, we would expect the value of ϕ to be about 100 nsec, similar to that of free DNS-σ. The observed ϕ value of 205 nsec suggests that only a fraction of σ molecules has been released. For a system containing both free and bound σ, since $S(t)$ is the same for DNS-σ in both free and bound states, the following relationships hold, according to Weber's law of addition of polarization (23)

$$F_y(t) = \frac{S(t)}{3}[f_a(1 + 2A_0e^{-t/\phi_a}) + f_b(1 + 2A_0e^{-t/\phi_b})] \quad [5]$$

$$F_x(t) = \frac{S(t)}{3}[f_a(1 - A_0e^{-t/\phi_a}) + f_b(1 - A_0e^{-t/\phi_b})] \quad [6]$$

where ϕ_a and ϕ_b are the rotational correlation times, and f_a and f_b are the fractions of free and bound σ, respectively. Using ϕ_a = 103 nsec and ϕ_b = 800 nsec for free DNS-σ and DNS-σ bound to the enzyme–DNA complex, respectively, a series of theoretical emission anisotropy curves was generated for various combinations of f_a and f_b over the time period comparable to that used to determine the experimental anisotropy (Fig. 4). Although these curves are somewhat nonlinear, their deviation from linearity is not large enough to preclude application of the linear least squares analysis. Thus this analysis was used to obtain the corresponding values of the rotational correlation time.

Comparison of the experimental data in Fig. 3 with the theoretical anisotropy curves indicates that 55% of DNS-σ was released from the enzyme–DNA complex due to RNA synthesis. We have further examined conditions for σ release and the results are summarized in Table 1. Release of σ did not occur if rifampicin was added prior to addition of four nucleoside triphosphates to block RNA chain initiation, indicating that the initiation was necessary for σ release. However, addition of three nucleoside triphosphates, which was likely to initiate short RNA chains, was not sufficient to induce significant σ release. Furthermore, the released σ was accessible to the added core enzyme. This was shown by a decrease in the fraction of free σ from 55% to 15% when



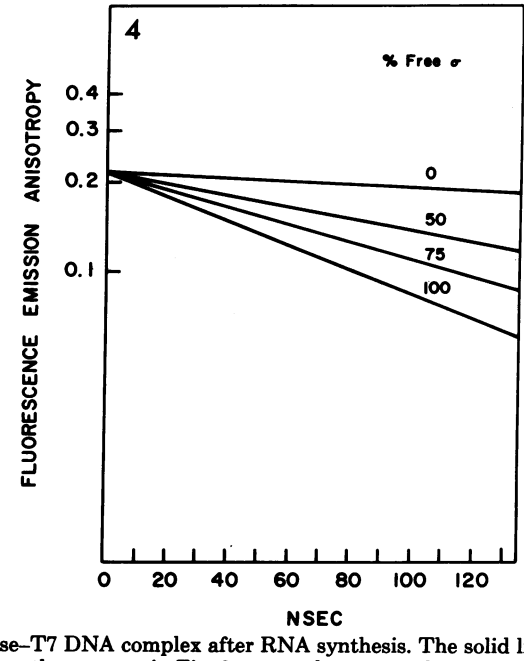


FIG. 3. Time-dependent emission anisotropy of the DNS-σ–core polymerase–T7 DNA complex after RNA synthesis. The solid line is a theoretical plot for ϕ = 205 nsec and A_0 = 0.21. The sample and conditions were the same as in Fig. 2 except that 69 nmol of T7 DNA and 150 nmol each of ATP, GTP, CTP, and UTP were added. The incubation was 5 min at 37° followed by 10 min at 23° prior to data collection.

FIG. 4. Theoretical emission anisotropy curves for various mixtures of free and bound DNS-σ. The method for calculating these curves was described in the *text*.

Table 1. Release of DNS-σ during transcription*

Conditions	ϕ_{obs} (nsec)	% of Free DNS-σ
DNS-σ	103	100
DNS-σ + Core E	599	0
DNS-σ + Core E + T7 DNA	800	0
+ U, G, C	681	5
+ Rif + A, U, G, C†	806	0
+ A, U, G, C	205	55
+ Rif + Core E‡	441	15
+ Core E‡	237	45
DNS-σ + Core E + fd DNA	508	
+ A, U, G, C	298	

* The experimental conditions were as described in Fig. 3. The following amounts were used: (in 0.5 ml) DNS-σ, 35 pmol; core polymerase (E), 35 pmol; T7 DNA, 69 nmol; fd DNA, 80 nmol; nucleoside triphosphates (A, U, G, C), 100 nmol; and rifampicin, 1.2 nmol.

† The DNS-σ–core polymerase–T7 DNA complex was incubated with rifampicin for 5 min at 23° before addition of nucleoside triphosphates.

‡ Experiment was performed after σ was released by RNA synthesis. When indicated, the reaction mixture was incubated with rifampicin for 5 min at 23° before addition of core polymerase (35 pmol).

core polymerase was added after σ was released in the presence of four nucleoside triphosphates. In this experiment, addition of rifampicin before core polymerase was necessary to prevent the reinitiation of RNA chains. In the absence of rifampicin, little decrease in free σ (from 55% to 45%) was seen after addition of core polymerase. Presumably the released σ which had been taken up by added core enzyme was released again as a result of reinitiation. Unlike the σ specificity, the σ release was not confined to native double-stranded DNA. It also occurred with single-stranded DNA such as phage fd DNA. This was shown by the decrease in the rotational correlation time for the DNS-σ–core polymerase–fd DNA complex from 508 nsec in the absence of nucleoside triphosphates to 298 nsec in the presence of all four of them (Table 1).

A question which may be raised here is why only about 50% of σ was released after starting RNA synthesis? To answer this question we have studied the initiation of RNA chains on T7 DNA by RNA polymerase, measuring [γ-^{32}P]ATP incorporations into 5′ terminal nucleotide of RNA product (24) under the conditions similar to that used for the σ-release experiments (Table 2). For both unmodified holoenzyme and the DNS-σ–core polymerase complex, we calculated that about 0.4 to 0.5 RNA chains were initiated per enzyme molecule. This implies that about 50% of holoenzyme was active in transcription, in good agreement with the observation that only about 50% of σ was released under similar conditions.

DISCUSSION

By means of nanosecond fluorescence depolarization spectroscopy we have demonstrated physical release of σ from the RNA polymerase–DNA complex due to RNA synthesis and subsequent take-up of the released σ by core polymerase. Under low salt conditions, the stoichiometry of σ release is consistent with the stoichiometry of the enzyme that initiates RNA chains. Evidence that σ may also be released *in vivo* has been provided by the observation that the folded bacterial genome isolated by gentle lysis of the cell does not contain σ, yet has many actively polymerizing core polymerase molecules (25).

Table 2. Initiation of RNA chains on T7 DNA by RNA polymerase*

Enzyme	Conditions	[γ-^{32}P] ATP incorporated (pmol)	RNA chains initiated (pmol)	RNA chains initiated per enzyme molecule
σ + Core E	5 min, 37°	3.0	4.5	0.38
DNS-σ + Core E	5 min, 37°	2.7	4.1	0.34
	+ 15 min, 23°	3.6	5.4	0.45
	+ 30 min, 23°	3.5	5.3	0.44

* Reaction mixtures (0.1 ml) contained 0.05 M Tris (pH 7.8), 0.05 M KCl, 0.01 M $MgCl_2$, 10^{-3} M dithiothreitol, 3×10^{-4} M each of UTP, GTP, and CTP; 1.6×10^{-4} M [γ-^{32}P]ATP (1880 cpm/pmol); 2.3 nmol of T7 DNA, 12 pmol of DNS-σ, and 12 pmol of core polymerase. Reactions were performed as described in *Materials and Methods* for the indicated time periods. Number of RNA chains initiated was calculated based on the ATP/GTP ratio of 2/1 for the initial 5′ terminal nucleotide (30).

The blockage of σ release by rifampicin clearly indicates that RNA chain initiation is required for the release. However, the failure of three nucleoside triphosphates to cause significant amount of σ release suggests the σ does not release during the initiation process itself, but after the RNA chains have reached a certain length. In accord with this observation is the finding by Krakow and Fronk (26) that by electrophoresis σ could not be separated from the RNA polymerase–poly[d(A-T)] complex during the formation of the first few phosphodiester bonds, whereas σ separation was achieved during poly[r(A-U)] synthesis.

σ release during transcription was also observed with a single-stranded DNA template, fd DNA. An unusual finding here was the decrease in the value of ϕ for the DNS-σ–core polymerase complex from 599 nsec to 508 nsec when fd DNA was added. This could be interpreted as the release of a small amount of σ (<15%) by single-stranded DNA (but not seen with double-stranded DNA such as T7 DNA). Alternatively, since holoenzyme exists as a dimer under low salt conditions (2), the decrease in ϕ might reflect the template-induced dissociation of RNA polymerase dimer into monomers (27). If the latter is the case, the observed ϕ value of 508 nsec suggests that the dissociated monomeric enzyme is still bound to the DNA template because the value of ϕ for a free monomeric DNS-σ–core enzyme complex would be about 300 nsec.

It should be mentioned that rotational correlation times of proteins determined by nanosecond depolarization spectroscopy are usually about twice the values calculated for rigid, unhydrated spheres of equivalent molecular weights (28). The ratios of the observed and calculated rotational correlation times for DNS-σ and the DNS-σ–core polymerase complex are 3.8 and 2.3, respectively. These ratios could be attributed to hydration, asymmetry, and aggregation of the protein molecules. Detailed analysis of the observed rotational correlation times in terms of size and shape of σ and holoenzyme molecules will be presented elsewhere (Yarbrough and Wu, to be published)

The mechanism of σ release is not known. There are two ways that σ release could occur. (*a*) The conformation of RNA polymerase may be altered when it is in the process of

synthesizing RNA chains. Such a conformational change could reduce the affinity of σ for core polymerase. To result in σ release, this reduction in affinity must be rather large, since the K_d for the σ-core polymerase complex is less than 10^{-9} M (29). (*b*) The release may be due to physical displacement of σ by the growing RNA chain. It has been reported that the binding of holoenzyme to single-stranded polynucleotides is sufficient to promote the release of σ when release is assayed by gel electrophoresis (26) but not when release is assayed by sucrose gradient sedimentation (8). In a previous report (29) we have shown that less than 25% of σ bound to core polymerase is released by addition of tRNA or poly(U), using nanosecond fluorescence depolarization spectroscopy. Thus it is likely that the affinity of core polymerase for σ is reduced by RNA product but other driving forces such as the conformational change of enzyme described above are needed to induce physical release of σ.

A potential capability of nanosecond fluorescence depolarization spectroscopy is the detection of segmental flexibility of macromolecules (28). The shortening of the rotational correlation time of the DNS-σ–core polymerase–DNA complex during transcription without a marked change in the linearity of the time-dependent anisotropy plot (Fig. 3) indicates that we are not observing some local flexibility or increased mobility of σ within the enzyme–DNA complex as a result of RNA synthesis. Such information would not have been resolved by steady-state fluorescence spectroscopy.

In conclusion, we have successfully used nanosecond fluorescence depolarization spectroscopy to demonstrate physical release of σ during *in vitro* transcription. It should be emphasized that this technique can also be applied to characterize other biologically important dynamic processes which involve association-dissociation of macromolecular complexes.

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