Kinetic measurement of ribosome motor stalling force

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We measure the ribosome motor stalling forces to unzip mRNA polymers during gene expression. An approach of using the changes in the reaction rate constants to determine the molecular motor forces is presented. Specific antisense DNA oligomers complementary to mRNA templates are used as kinetic barriers for estimating the ribosome forces using real time bioluminescence detection of luciferase gene expression. The rate constants are determined by comparing the experimental data with numerical simulation of gene expression to deduce the ribosome force (26.5 ± 1 pN) required to unzip mRNA polymers. Understanding the forces generated by the ribosome may also enable the construction of information-based artificial nanoscale machines. © *2004 American Institute of Physics*. [DOI: 10.1063/1.1821647]

Unzipping DNA and mRNA polymers is central to accessing and regulating genetic information.[1] It is also essential in recreating artificial circuits on functional biochips for applications in nanometer scale technology. Recent single DNA molecule studies have measured the forces involved in separating the double strands of DNA,[2] the processivity and stalling force of RNA polymerase during DNA transcription,[3,4] and the rupture forces that stabilize biomolecular structure and interactions.[5,6] A theoretical framework for the unzipping mechanism has also been explored.[7,8] Single molecule analysis[9] combined with the recent determination of ribosome structure[10] point toward the possibility of studying the forces involved in this complex molecular machine. The ribosomal forces exerted to unzip mRNA are crucial in regulating the dynamics of gene expression.[11] However, unlike DNA polymers, mRNA is very unstable and the ribosome is a multi-subunit complex. Thus single molecule force measurements on the ribosome are difficult and have not been attempted.

In this letter, we present an approach of using the ribosome motor activity to determine its translation (or stalling) force. In this method, we create an energy barrier of known strength using single stranded DNA oligomers (also referred to as antisense probes) hybridized to mRNA, and monitor the changes in the kinetics of gene expression. These artificial barriers are designed to mimic the inherent mRNA topological kinetic constraints due to its secondary structure. We manipulate the strength of the energy barrier for ribosome motor moving on mRNA by using oligomers of different length and complementary to mRNA at specific position along the mRNA sequence. We study the changes in the net rate of translation as a function of the oligomer length and sequence specificity using bioluminescence detection. Comparison of our experimentally measured data with simulation of gene expression enables us to deduce the stalling forces that a ribosome can impart to break open the mRNA secondary structure.

Our experimental design, to measure ribosome forces, is based on the interplay between connecting a thermodynamic quantity to molecular mechanics. In the transition state theory, the rate of a chemical reaction is given by

$$k = k' e^{-\Delta G/K_B T}, \tag{1}$$

where ΔG is the free energy change. Single molecule experiments on molecular motors have exploited this approach to directly measure the molecular force and relate it to the relevant thermodynamic quantities.[12] In our method, we impose a physical energy barrier (using oligomers) for ribosome molecular motor and measure the changes in the rate of protein production. The ribosome has to do extra work "W," other than translating mRNA, in order to move across the barrier and as a result the rate of translation is decreased. The two rates of translation can be related by

$$k_{\text{oligo}} = k_{\text{translation}} \exp^{\{-(\Delta E - W)/k_B T\}}, \tag{2}$$

where $k_{\text{translation}}$ is the rate of translation when no oligomer is added to the *in vitro* translation system, k_{oligo} is the rate of translation when an oligomer of length δl is added to *in vitro* translation system, ΔE is the hybridization energy of mRNA and DNA oligomer, W is the work done by the ribosome to overcome the barrier ΔE, and K_B is the Boltzmann constant. Since the base sequences are known, the binding energy is calculated by counting the nearest-neighbor interaction energies, expressed in terms of kcal/mol (0.6 kcal/mol=1 $k_B T$). Using the measured values of $k_{\text{translation}}$, k_{oligo}, and ΔE in Eq. (2) one can find W. Here $W = F\delta l$, where F is the force exerted by the ribosome and δl is the length of the oligomers. The DNA oligomer-mRNA hybrid, which is a stable structure, allows one to estimate the binding energy and thus the barrier height more accurately. A schematic of our experimental design is shown in Fig. 1. The folded secondary structure of the luciferase gene sequence, using the mRNA folding algorithm,[13] is shown in Fig. 1(a). Single stranded n-mer DNA oligomers (where n is 30, 45) and of different

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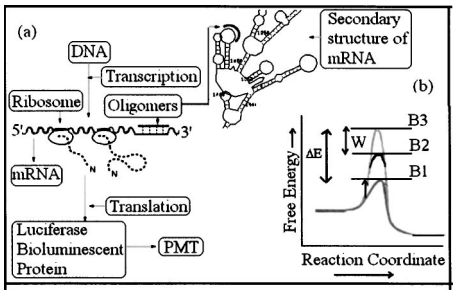

FIG. 1. Schematic of the experiment: (a) Antisense oligomers are used to create an energy barrier for the translating ribosome; these barriers mimic the secondary structure of mRNA. (b) A pictorial representation of the energy barriers seen by the ribosome prior to and after oligomer hybridization: B1 is the barrier created by the secondary structure of mRNA; B3 is the extra barrier that is created by the oligomer and mRNA hybridization. B2 is the effective barrier that is seen by the ribosome after doing active work W on hybrid. ΔE is the free energy of oligomer-mRNA hybridization.

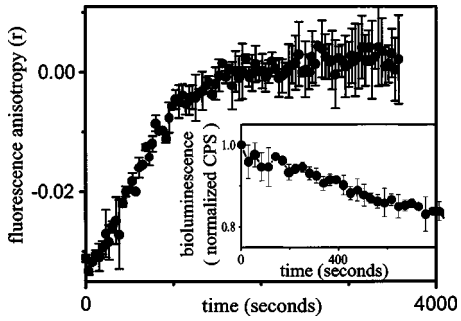

FIG. 2. Effect of specific oligomers on transcription and bioluminescence kinetics: Real time measurement, using fluorescence anisotropy, of transcription kinetics is plotted. The data are an average of six-transcription kinetics measurements (three without oligomers and three with oligomers) are plotted (closed circles) with error bars. In the inset, average of four (two with oligomers and two without oligomers) luciferase kinetics measurement (closed circles) is plotted with error bar.

sequence composition were designed by comparing the sequences with the accessible sequences in the mRNA structure. The details of the oligomers and their thermodynamic parameters are given in Table I. A schematic of the kinetic barrier seen by the ribosome motor is shown in Fig. 1(b).

We use TNT® T7 Coupled Wheat Germ Extract System—WGES from Promega for studying the kinetics of *in vitro* transcription coupled translation of luciferase gene.[14] 1 μl of antisense oligomers of three different lengths (1.3 mM concentration) was added in the reaction mixture to create kinetic barriers during translation. The effective oligomer concentration added in the three cases is similar. The oligomers are in excess such that they hybridize to the newly synthesized mRNA templates. The real-time gene expression was monitored using 1 μl of 1 mM luciferin added to the 20 μl reaction volume.[15] We established experimentally that the oligomers added in the reaction mixture affect only the rate of translation by measuring independently the rate of transcription using fluorescence anisotropy[16] (Fig. 2) and the luminescence kinetics[17] (inset to Fig. 2).

A confocal fluorescence anisotropy setup is used to test the effect of oligomers on transcription kinetics in a 20 μl reaction mixture. The details of the experimental setup are presented elsewhere[18] We followed the standard protocol of transcription (Promega) except that we used a mixture of fluorescent-labeled (Alexa-488, Molecular probes) and unlabeled r-UTP (1:10 ratio). The anisotropy $(r=(I_{\parallel}-I_{\perp})/(I_{\parallel}+2\times I_{\perp}))$ of the labeled r-NTP was monitored in real time to deduce the transcription kinetics. Transcription is a polymerization process of the nucleotides, and the fluorescent-labeled nucleotides are incorporated into the growing polymer chain. As the polymerization proceeds, the anisotropy of the fluorophores in the polymer increases compared to that of the free monomers (Fig. 2). To test the effect of oligomers on bioluminescence, we *in vitro* synthesized luciferase protein and measured its luminescence by adding luciferin in the presence and absence of oligomers (Fig. 2 inset).

In Fig. 3, we plot the luminescence (photons/second) versus time of luciferase gene expression kinetics. The measured luminescence is directly related to the rate of protein synthesis. We plot the length dependence of the oligomer (0, 30, and 45 bases) bound to the nascent mRNA template on gene expression kinetics. The length of the oligomer tunes the strength of the kinetic barrier. The sequences are designed such that the starting base position is unchanged. As the effective length of the oligomer is increased the kinetic barrier for ribosome translation is enhanced. A minimum of five data sets, for each oligomer parameter, is measured to estimate the error bars in the experiment. In the inset of Fig. 3 we show the specificity of the oligomer-mRNA hybridization on the gene expression kinetics. As seen in the data (Fig. 3 inset), we find that the nonspecific sequence does not make

TABLE I. Characteristics of mRNA oligomer hybridization.

Sequence	BE (k.cal/mol)	$k_{translation}$ (/sec) (input rate)	Force (pN)
No oligomers	···	0.106	
TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT (nonspecific 30 mer)		0.085	
ACCGCTGGAGAGCAACTGCATAAGGCTATG (specific 30 mer)	38.8	0.041	~26.3
ACCGCTGGAGAGCAACTGCATAAGGCTATG AAGAGATACGCCCTG (specific 45 mer)	61.4	0.0025	~27.1
ACCGCTGGAGAG**G**AACTGCATAAGGCTATG (specific 30 mer with one base mutation)	39.3[a]	0.051	
ACCGCTGGAGA**TGT**ACTGCATAAGGCTATG (specific 30 mer with three base mutation)	40.3[a]	0.009	
ACCGCTGGAG**TATGG**ACTGCATAAGGCTATG (specific 30 mer with five base mutation)	39.7[a]	0.025	

[a]Value predicted from our model, taking ribosomal force to be 26.7 pN.

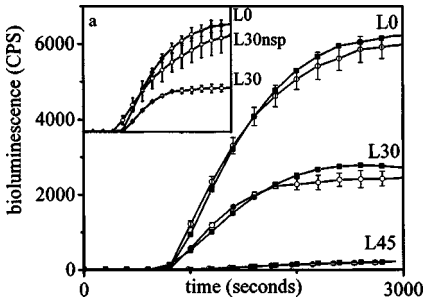

FIG. 3. Effect of length and specificity of antisense oligomer on translation. Bioluminescence of luciferase is plotted as a function of time for an *in vitro* expression system. Kinetics of luciferase production is seen to change when specific oligomers of different lengths are added in the expression system. L0 is the kinetic measurement of luciferase production when no oligomer is added, L30 when specific oligomer of length 30 nucleotides is present, and L45 with specific oligomer of length 45 nucleotides in the expression system. In the inset we compare the kinetic measurement of luciferase production in presence of specific (L30) and nonspecific oligomer (L30nsp) of the same length. Comparison of experimental results (open circle) to simulation (closed square) is also shown.

a large contribution to the kinetic barrier for ribosome translation. By comparing our data of the specific versus nonspecific hybridized oligomers, we can show that the oligomers participate only in tuning the effective kinetic barriers in translation. We have simulated gene expression kinetics to estimate accurately the changes in translation rate with and without adding the antisense oligomers.[19,20] The fits to the simulation are shown as closed squares in Fig. 3 and the changes in the translation rates are given in Table I.

To deduce the binding energy, we use the nearest-neighbor model.[21] In this model the hybrid duplex initiation is assigned one $\Delta G'$ value and the ΔG of the propagation depends on the sequence of nearest-neighbor pair seen during propagation (for example, $\Delta G = -1.8$ kcal/mol for RNA/DNA hybrid formation rAG/dTC pairing and -1.3 kcal/mol for rGA/dCT pairing). ΔG for the initiation of any such pairs is 3.1 kcal/mol. Therefore to calculate the binding energy of DNA/RNA duplex hybrid, we count each nearest-neighbor pair in the hybrid region and sum the ΔG values of all nearest-neighbor pairs to the ΔG value for hybrid duplex initiation. The calculated values of the binding energy for 30-mer and 45-mer DNA-mRNA hybrids are given in Table I. The translation forces required by the ribosome to overcome such barriers is then given by

$$F = \frac{\Delta G + K_B T \ln\left(\frac{k_{\text{specific}}}{k_{\text{nonspecific}}}\right)}{l}, \tag{3}$$

where l is the length of the oligomer. Using the values from Table I, we find the ribosome stalling force is 26.5 ± 1 pN. Interestingly this value compares well with single molecule force measurements of RNA polymerase motors.[3,4] We then use the value of the ribosome force and the changes in the translation rate to predict the binding energy of the oligomers with specific mutations hybridized to mRNA. We observe that one-base or three-base mutations enhance hybrid formation (Table I) suggesting that specific secondary structures are more favored when there are mutations. We find that the large change in protein production rates for no oligomer (input rate $= 0.12$ per s) and with 45 base-oligomer (input rate $= 0.004$ per s) corresponds to a very small change in the energy barrier per base-pair. In our case the calculated binding energy per base-pair distance is of the order of 26 pN, and indeed the measured force deduced from the changes in the rate of translation is close to this estimate. These observations imply that marginal changes in free energy of secondary structures lead to large changes in protein production rates.

Very little is known about the mechanism by which a ribosome breaks the secondary structure during translation. The magnitude of the force that we estimate (26.5 ± 1 pN) suggests that the ribosome, unlike other molecular motors such as RNA polymerase (stalling forces of 12–25 pN[3]), myosin (3.5 pN[22]), and kinesin (5–6 pN[23]) is highly processive. Our estimate gives an average force that the ribosome exerts during translation to unzip mRNA polymers. Clearly this estimate puts an important limit to the size of the secondary mRNA local structure.

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[15] Luciferase gene is driven by a T7 promoter. The total reaction volume is 20 μl and contains all the required ingredients (T7 RNA polymerase, the ribosome, the nucleotides, the amino acids, the luciferase gene, and luciferin) for the production of the luciferase proteins. Luciferase protein catalyses luciferin to oxy-luciferin, which result in the emission of photons. The data from the PMT detector is recorded using an AT-MIO-16XE10 (National Instruments) data acquisition board and LabVIEW.
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