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**PHYSICS of LIFE
reviews**

Physics of Life Reviews 6 (2009) 250–266

www.elsevier.com/locate/plrev

Review

DNA based molecular motors

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Received 23 September 2009; received in revised form 23 September 2009; accepted 23 September 2009

Available online 3 October 2009

Communicated by L. Perlovsky

Abstract

Most of the essential cellular processes such as polymerisation reactions, gene expression and regulation are governed by mechanical processes. Controlled mechanical investigations of these processes are therefore required in order to take our understanding of molecular biology to the next level. Single-molecule manipulation and force spectroscopy have over the last 15 years been developed into extremely powerful techniques. Applying these techniques to the investigation of proteins and DNA molecules has led to a mechanistic understanding of protein function on the level of single molecules. As examples for DNA based molecular machines we will describe single-molecule experiments on RNA polymerases as well as on the packaging of DNA into a viral capsid—a process that is driven by one of the most powerful molecular motors.

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PACS: 82.35.Lr; 82.37.Np; 82.37.Rs; 82.39.Pj; 87.15.H

Keywords: Single-molecule; Optical tweezers; RNA polymerase; Molecular motor; DNA packaging; DNA overstretching; Pol II; Bacteriophage

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1. Introduction

DNA molecules are the carrier of all genetic information and have evolved both, as extremely stable entities in order to preserve this genetic code, as well as in ways that allow for the controlled access of this information. Thus in addition to understanding the biochemical information of a DNA molecule, it is also important to study its mechanical behaviour and characteristics, since it is these characteristics that control many of the biological processes that take place during gene expression.

To this end, mechanical studies of single biomolecules have in recent years revolutionised our understanding of biological complexes [1–6]. The most prominent methods for single-molecule force spectroscopy are the atomic force microscope (AFM), magnetic tweezers and optical tweezers [7]. In single-molecule force spectroscopy AFMs have mainly been used for experiments which require high forces, i.e. to rupture bonds or unfold proteins [8]. Magnetic tweezers allow for the study of proteins that twist DNA and proteins that are very sensitive to forces [9]. Optical tweezers are probably the most widely used tool for studying the mechanics of molecular motors [10]. It has been shown that forces as low as ~ 10 fN [11] and distance changes as low as 0.1 nm [12–14] can be observed using optical tweezers. Moreover, with the use of clever experimental geometries [15] or strong lasers, it is possible to exert forces exceeding 100 pN. These forces and distances are in the range reported for molecular motors [16,17].

One of the most important applications of single-molecule force spectroscopy is to unravel the mechanics of DNA molecules [18,19]. For any DNA the measured force-extension behaviour can be well described (up to forces of about 50 pN) using a simplistic coarse grained polymer model. Moreover, at high forces or in situations when the DNA helix is overwound (i.e. the number of bases per complete turn of the DNA helix is reduced) or underwound, the conformation of the DNA changes completely from the canonical B-DNA structure to a different, stable form in a spontaneous and cooperative manner which resembles a phase transition.

Understanding the mechanical behaviour of single DNA molecules becomes biologically important in the context of DNA–protein interactions. Proteins that bind DNA or translocate along DNA oftentimes exert forces on the DNA substrate due to bending or unwinding [20,21]. Typical forces that are applied are in the range of 1–100 pN and typical displacements that need to be observed can be as small as a single basepair (bp) which is only 0.34 nm. Physically one can understand these force- and distance-scales since the energies important for understanding the underlying phenomena lie between the thermal energy $k_B T$ which in units of force times distance equals to 4.1 pN nm and the energy available through the chemical reaction. In the case of ATP hydrolysis at physiological conditions the free energy liberated by the hydrolysis of a single ATP molecule by a single protein amounts to ~ 100 pN nm for the hydrolysis. The time-scales that are important to monitor such processes reach from the millisecond to second regime. Single-molecule manipulation with optical (or magnetic) tweezers allows for investigations at these force-, distance-, and time-scales. Hence, optical tweezers are an ideal tool for the mechanistic investigations of these protein nucleic acid interactions at the single-molecule level. The high sensitivity of the single-molecule experiments allows for a detailed analysis of this enzymatic behaviour and opens up the possibility to unravel the underlying molecular mechanisms. A huge variety of such DNA–protein interactions have been studied using single-molecule manipulation techniques and the results are summarised in recent reviews [9,22]. Of particular interest are proteins that use the free energy liberated by a chemical reaction (most often the hydrolysis of ATP) to either translocate along DNA [22], unwind DNA [23], change the topology of DNA [24] or catalyse a polymerisation reaction [3,25], thus constituting molecular motors.

In the literature one can find many excellent reviews on optical trapping and single-molecule force spectroscopy [10,26–29] and the purpose of this article is not to give another complete overview of the field. Instead, we focus on some key aspects of current research and discuss experimental procedures as well as current challenges (Section 2). In particular we describe in detail the design of an optical tweezers apparatus that allows studying the mechanical properties of DNA molecules, such as the well-known force-extension behaviour and the overstretching transition (Section 3). This is a necessary pre-requisite for investigating the molecular mechanism of molecular motors that

Table 1

Comparison of single-molecule force spectroscopy methods.

	Force resolution (pN)	Maximum force (pN)	Resolution (nm)	Spring constant (pN/nm)	Thermal bandwidth (Hz)	Useful references
AFM	5	10 000	<0.1	$10\text{--}10^5$	10 000	[8,81,116]
Magnetic tweezers	0.001	30	2–10	10^{-6}	10–100	[7,117]
Optical tweezers	0.1	150	0.1–2	0.01–1	1000–10 000	[10,28,29]

translocate along DNA. We will review two examples of DNA based molecular motors, namely DNA packaging by a viral portal motor complex (Section 4) and transcription by RNA polymerases (Section 5).

2. Single-molecule force spectroscopy

While over the years many different single-molecule force spectroscopy techniques have been developed in this review we will focus on the three most common approaches, the atomic force microscope (AFM) [8], magnetic tweezers [7], and optical tweezers [29]. There are many similarities between these techniques. In each case, single molecules are manipulated by attaching one end of these molecules to a mechanical attachment point such as a micron-sized bead (in the case of magnetic and optical tweezers) or an AFM cantilever. The other end is then attached to the surface of a coverslip (though optical tweezers allow an interesting alternative which we discuss further below).

Once the molecule is attached it can be stretched by separating the attachment points—typically with piezoelectric actuators. In some experiments the force is constantly increased until a certain level is reached or until a molecular bond is ruptured. This is known as a constant *force ramp*. Other experiments require that the force exerted on the molecule remains constant during the experiment, the *constant force* mode. These are the most common force control protocols, but others have also been developed such as the force jump [30] or the so-called passive [31] or semi-passive modes [32]. Moreover, there are now ways to relate measurements collected in different force configurations [33]. In all of these cases, it is crucial to properly calibrate the force, and we will review how forces are calibrated in the three devices and compare their experimental capabilities.

Despite their similarities, these techniques have crucial differences. The force ranges easily applied by each technique differs over several orders of magnitude, with forces as small as ~ 0.1 fN easily applied in a controlled fashion via magnetic tweezers to forces as large as several nN applied with AFMs. In addition, the detectable distance-scales similarly vary with AFMs having the greatest sensitivity, ~ 0.1 nm, to magnetic tweezers with the worst, ~ 10 nm. Finally, the time-scale of the fastest measurable biological activity also differ. Physical limitations restrict typical magnetic tweezers measurements to ~ 10 ms while optical tweezers and AFMs can, in principle, observe motions as fast as ~ 0.1 ms. Table 1 summarises these useful rules of thumb for typical performance of these different techniques. In practice, the intermediate distance-, force-, and time-scales afforded by optical tweezers measurements are the most similar to biological processes—nanometer-scale motions under loads of tens of pN on the millisecond time-scale—thus, optical tweezers have proven the most versatile in biological measurements.

2.1. Force spectroscopy with an AFM cantilever

AFM cantilevers are well known from imaging applications. The principle of using an AFM cantilever as a tool for single-molecule force spectroscopy is fairly simple. By illuminating the backside of the cantilever with a powerful but incoherent light source (typically a super-luminescent diode) and detecting the reflection of this light signal using a position sensitive detector (such as a quadrant photodiode or a position sensitive diode) the position of the cantilever tip can be monitored. If a force is exerted on the cantilever tip, the cantilever bends and this bending is measured with the detector. Due to the small dimensions of the cantilever on the order of 100 μm , the resulting spring stiffness is fairly low with effective spring constants ranging from 10^1 to 10^5 pN/nm. Calibration of the spring constant is typically achieved by measuring the power spectrum of the thermally driven motion of the cantilever, however, there are many experimental pitfalls especially when operating in viscous solvents [34].

In comparison to other single-molecule force spectroscopy approaches the spring constant of the cantilever is still fairly large. This limits the obtainable force resolution to about 10 pN. In return, the spatial resolution of the cantilever is extremely good exceeding the 0.1 nm limit. Thus AFM cantilever have been used in particular to monitor

the rupture of bonds such as those between receptor and antibody [35], interactions between molecules and surfaces [36], unfolding of proteins due to the rupture of hydrogen bonds, salt bridges, etc. [37] and even to rupture covalent bonds [38–40]. By experimentally varying the force loading rate, a technique commonly referred to as dynamic force spectroscopy, it is possible to unravel details of the underlying energy landscape such as the height of the energy barrier and the distance to the transition state [41–43]. Besides the rupture of bonds it is also possible to simply stretch molecules in a repetitive fashion thus unravelling the force-extension behaviour e.g. of DNA [44] or RNA molecules, polypeptide chains [45], spectrin [46] and fibrin [47] fibers and membrane proteins [4] among many others.

In addition it has been shown that force-feedback circuits can be constructed for AFMs in order to hold molecules at a constant force [48]. However, due to the limited force resolution of the AFM such a feedback is only possible at elevated forces of $\sim 10\text{--}20$ pN and above, where most molecular motors have already stalled.

2.2. Force spectroscopy using magnetic tweezers

The experimental design of a magnetic tweezer is fairly simple. A super-paramagnetic bead is drawn into the highest gradient of a magnetic field created by typically two permanent magnets. The motion of the bead is imaged onto a CCD camera via brightfield microscopy. The interference pattern that develops between light reflected from the nearby surface and from the bead can be exploited to sensitively measure the distance of the bead from the surface. By changing the position of the magnets with respect to the bead, the applied force can be changed. In parallel, the magnets can also be rotated, allowing for the application of torque to the molecule under study. Due to the relatively low bandwidth and spatial resolution of the detection techniques used in typical magnetic tweezers, the distance resolution is much lower than in optical tweezers or AFM. However, this limitation is an instrumentation issue only. It should be possible to use laser detection methods, as is done with optical tweezers, and obtain the same high spatial resolution, albeit with a different thermal bandwidth (Table 1).

There are three important features of magnetic tweezers which make them the method of choice for a variety of different applications. First, the very low spring constant of a magnetic tweezers of typically 10^{-6} pN/nm gives the opportunity to easily perform measurements at constant force, since movements in the range of 1 μm only result in changes of forces on the order of 0.001 pN. Second, by rotating the magnets in a magnetic tweezers and using attachments of the biomolecules which consist of many linkages, torsion can be exerted in a controlled manner. Of course this requires the molecule to be torsionally constrained—i.e. it must have multiple attachment points so that it is not free to swivel around one of them, releasing the torque. The ability to apply torque is of particular importance for experiments aimed at investigating proteins that alter the topology of the DNA by introducing supercoils [19,49]. Third, since measurements merely amount to tracking of the interference patterns of the magnetic beads, it is possible to monitor multiple experiments simultaneously in a single field of view, and thus allowing for a highly parallel data acquisition. This is extremely important for measurements where the activity of the particular enzyme is rather low, which is the case for certain RNA polymerases [50], or when force measurements are combined with single-molecule fluorescence measurements [51] where the amount of useful collected data is reduced since two different single-molecule techniques are performed in parallel. Magnetic tweezers have been employed to characterise the mechanical properties of DNA and RNA molecules [19] as well as for the investigation of topoisomerases [52], DNA translocases [53], helicases [54], nucleosome remodelling complexes [55], viral packaging motors [51] and RNA polymerases [56].

2.3. Force spectroscopy with optical tweezers

Optical tweezers are by far the most versatile for investigating the behaviour of DNA based molecular motors. While the distance resolution of an optical tweezers can be as good as 0.1 nm, it is not quite up to par with that of an AFM. Despite the differences one can detect sub-piconewton forces and sub-nanometer motions, permitting detailed experiments on enzymes where step-sizes down to a single bp are to be expected and forces need to be controlled on the pN level. In fact (almost) all the DNA based molecular motor experiments described in this review were obtained using optical tweezers and while excellent technical reviews about optical tweezers exist [29,57,58], we consider it important to familiarise the reader with the technique, experimental procedures and challenges.

In optical tweezers a three-dimensional gradient in light intensity is used to trap small dielectric objects near the focus. For objects larger than the wavelength of light this effect can easily be understood using wave optics and diffraction. Diffraction causes a change in momentum of the light transmitted through the dielectric object and

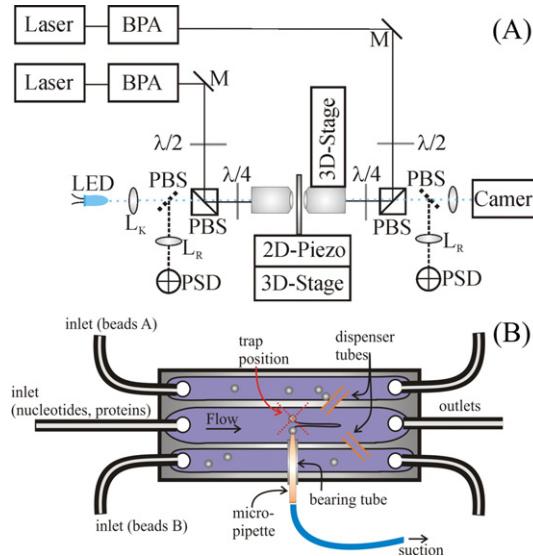


Fig. 1. Schematics of dual-beam optical tweezers. (A) Two counter-propagating diode lasers are used to form a dual-beam optical trap. The advantage of such a setup is the high stiffness, due to efficient trapping in the axial direction, and the possibility to determine the amount of transferred light momentum directly [15]. Abbreviations: BPA: beam profile adjuster, M: mirror, PBS: polarising beam-splitter, $\lambda/2$: half-wave plate, $\lambda/4$: quarter-wave plates, PSD: position sensitive detector, 3D-Stage: three-dimensional stepper motor driven position control, 2D-Piezo: two-dimensional linearised piezoelectric stage, LED: light emitting diode, L_R : relay lens. (B) The force spectroscopy experiments are performed within a micro-flow chamber consisting of three channels that are connected by dispenser tubes downstream from the experimental region. The upper and lower channels are used for the fast delivery of modified beads, while the central channel is used for tether assembly as well as for the actual experiments. A micro-pipette is glued to the chamber and acts as a mechanical anchor-point for microscopic beads which are attached to its tip via suction.

this change in momentum causes a restoring force to the object if not all intensities are balanced. The conceptually easiest way to measure the force applied by optical tweezers is thus to measure the changes in light momentum [15]. Practically this is often not possible since a high numerical aperture is required in order to form a stable trap in all three dimensions and thus some of the trapping light may be scattered to higher numerical aperture after diffraction, where it typically cannot be collected by the optical system of the microscope. Thus the total amount of transferred light momentum cannot be determined. This problem can be overcome by building a trap which consists of two counter-propagating lasers, similar to arrangements that are used in magneto optical traps (Fig. 1(A)). This so-called dual-beam optical tweezers apparatus has two characteristics that make it particularly useful for investigating DNA mechanics and DNA based molecular motors. First, the use of two counter-propagating beams leads to a very strong optical trap, capable of exerting forces well beyond the over-stretching regime. Secondly, it allows for the in-situ determination of the forces acting on a bead caught in the optical trap by direct measurement of the transferred light momentum [15] with a pN force resolution, far beyond what is possible with an AFM [44].

The system used for the experiments discussed here is similar to the one described by Smith et al. [15] and a schematic of the setup is shown in Fig. 1(A). The light of two diode lasers is each passed through a beam profile adjuster, comprised of astigmatism correction prism assemblies and a spatial filter, to produce circular Gaussian beams, which are then directed into the centre of a flow chamber. The light is focussed and collected with high NA objectives and detected on a pair of position sensitive detectors that monitor the image of the objective's back focal plane. The position of both, the right microscope objective as well as the sample chamber are controlled by two flexure stages and fine positioning of the chamber is achieved by linearised piezo-translators which can also be used to apply force-feedback. The two objective lenses are under-filled by the two counter-propagating laser beams. If the laser light is scattered by the object held at the centre of the trap, the high numerical aperture of the objective lens is used to collect all the scattered light (this holds for small deflections of the bead from the trap centre which typically correspond to forces up to ~ 100 pN), thus allowing for the direct measurement of transferred light momentum. Therefore, only calibration of the instrument's detection efficiency is required.

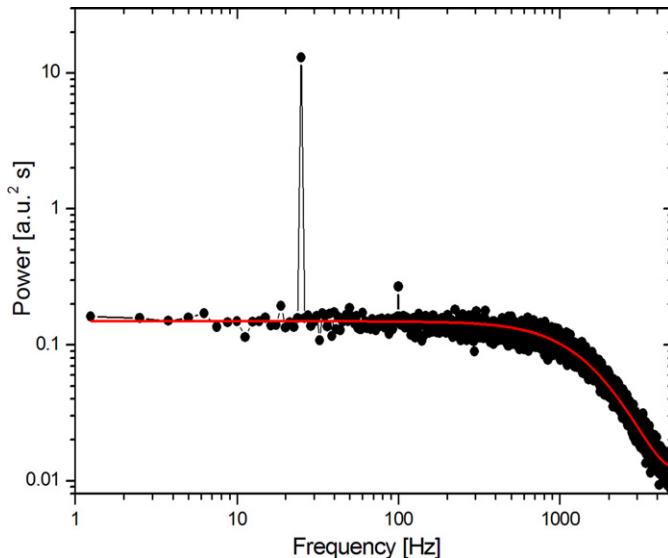


Fig. 2. Power-spectrum calibration of optical tweezers. The graph shows the averaged power-spectrum of the deflection signal recorded during trapping of a $\sim 1.9 \mu\text{m}$ diameter bead. The flow chamber was oscillated with a frequency of 25 Hz and an amplitude of $\sim 89 \text{ nm}$, resulting in a sharp peak in the power-spectrum. By fitting the amplitude of the peak in addition to the power-spectrum of the trap one can perform a direct calibration of the setup, which does not require a previous knowledge about the size of the trapped bead [59]. The fit determined a corner frequency of 1565 Hz, a spring constant of 0.18 pN/nm and from the diffusion constant D , a bead diameter of $1.9 \mu\text{m}$ can be determined using the Einstein relation ($D = k_B T / \gamma$, where γ is the drag coefficient of the bead), assuming a spherical bead size and a viscosity of the medium of 1 mPa s.

It is however, not necessary to measure directly the total amount of transferred light momentum since the detector signal is approximately proportional to the deflection, and by assumption of a harmonic trapping potential, also proportional to the force. The proportionality constants can be determined by calibration against known deflections and forces. In most applications of optical tweezers the thermal force is used. It causes the bead's deflection from the trap centre to fluctuate so that the motion of the bead is characterised by its drag coefficient and the stiffness of the trap. Unfortunately, both parameters are unknown and have to be determined experimentally, in addition to the constant that converts detector signal to deflection. Since three unknowns must be estimated in total proper calibration needs at least three known parameters.

The best way to calibrate the instrument is to record the thermal fluctuations of the bead and to simultaneously oscillate the micro-fluidic chamber using a piezo-electric stage. The stage is calibrated by capacitive sensors and run in a closed loop mode. The readout of the capacitive sensors therefore determines the amplitude of the oscillation. The power spectrum of the measured signal consists of two parts, a broad background caused by thermal fluctuations, and a sharp peak caused by the forced oscillations imposed on the bead by the motion of the micro-fluidic chamber (Fig. 2). By comparing the amplitude of that peak to the oscillation amplitude and fitting the corner frequency of the power-spectrum, one can determine the spring constant of the trap, the drag coefficient of the trapped object as well as the proportionality constant between the deflection of the bead from the trap center (measured in meters) and the detector readings (measured in arbitrary units) [59]. If one compares the forces determined by the oscillation/power-spectrum method to the measurement of momentum transfer one finds a good agreement within the experimental uncertainties of $\sim 5\%$. It should be noted that both methods can be used even if no *a priori* knowledge of the size of the trapped object is available, making them insensitive to variations in bead size.

The described methodology of measuring forces between a mechanical element in this case a micro-pipette tip and an optical trap has severe drawbacks. Mechanical elements are suspect to mechanical noise such as vibrations or thermal expansions. On the other hand precise position of the laser focus is altered by air density fluctuations that are constantly occurring. Techniques have been developed to overcome such problems. For example using detection lasers one can control the position of mechanical elements with 0.1 nm accuracy [60] and the position of the laser spot can be kept constant by enclosing the apparatus in helium atmosphere [13] or coupling the laser through single mode optical fibres and controlling the intensity that enters the microscope [61].

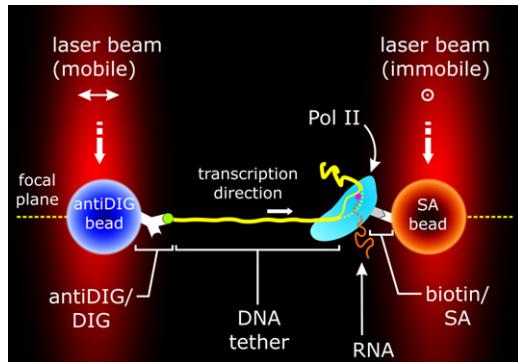


Fig. 3. Investigation of molecular motors in dumb-bell optical tweezers. As an example for an experiment in a dumb-bell optical tweezers a cartoon of a transcription experiment is shown. The polymerase is attached to a bead held in an optical trap. The end of the DNA tether is attached to a second bead held in a second optical trap. By manipulating the position of (at least) one of the optical traps the amount of load on the DNA molecule and thus on the polymerase can be adjusted. Different attachment chemistries e.g. DIG/anti-DIG on one side and biotin/streptavidin (SA) on the other are used to ensure specificity. In order to achieve the highest resolution the light for the two optical traps is coming from the same laser. The laser light is simply split into two orthogonal polarisations which can be read out separately allowing for precise differential back focal plane detection [63].

A more elegant and simple solution to these problems is to design the optical tweezers such that measurements are insensitive to these issues. The “dumb-bell” trap is such a design [13,62] (Fig. 3). By holding both ends of the biological system with two optical traps it is possible to make much of the instrument common between the two traps. Orthogonal polarisations can be used to form these traps and therefore the same laser and almost all of the optical components and optical path can be shared. The advantage is that any noise introduced by these components or by air fluctuations along the optical paths will be common to the two optical traps, producing identical fluctuations in both traps. Since the measurement is only sensitive to the distance between these traps, this extraneous noise is effectively decoupled from the measurement. Further improvements can be made by correlating the motion of each bead, allowing the separation of additional fluctuations [14,63]. Such a differential detection technique offers base-pair-scale resolution on the tens of milliseconds time-scale [32].

3. Mechanics of DNA and RNA molecules

Using single-molecule force spectroscopy one can manipulate single DNA molecules by tethering a fragment of DNA between two beads. For this purpose the two ends of the DNA have to be marked with different tags, e.g. one end can be biotinylated and the other labelled with digoxigenin (DIG), in order to bind to streptavidin and anti-DIG coated beads, respectively. Simple molecular biology techniques can be used to cut any plasmid or viral DNA, and introduce labelled nucleotides using a DNA polymerase [51] or labelled DNA oligomers in a ligation reaction. Due to its long length many experiments have been performed with the DNA from the bacteriophage λ [64].

Single-molecule force spectroscopy measurements in optical tweezers have to be performed in a serial fashion one molecule at a time, but information has to be extracted from histograms of many experiments. Therefore it is extremely important for the practicality of such experiments to employ micro-fluidic systems in order to allow for a fast turnover between experiments. A simple design was developed by Bustamante and co-workers [65] (Fig. 1B). With such a design one can assemble DNA tethers between two immobilised beads *in situ*. For this procedure a bead (coated with antibodies) is loaded into the lower flow channel, trapped by the optical tweezers and transferred to the end of a micro-pipette tip. The micro-pipette is glued into the flow chamber and beads are immobilised to its end using suction, thus providing a rigid mechanical attachment. Then, a second bead to which the DNA molecule is attached is caught by the optical tweezers near the outlet of the upper flow channel and brought into close proximity to the first bead. The strong affinity between antibodies attached to the bead and modified bases at the end of the DNA leads to the formation of a DNA tether, thus mechanically connecting the two beads. After tether formation, the molecule is stretched by moving the micro-pipette with respect to the position of the optical trap and the force-extension behaviour can be monitored (Fig. 4). For illustrative purposes a movie of this procedure can be found at www.cup.uni-muenchen.de/pc/michaelis/movies.

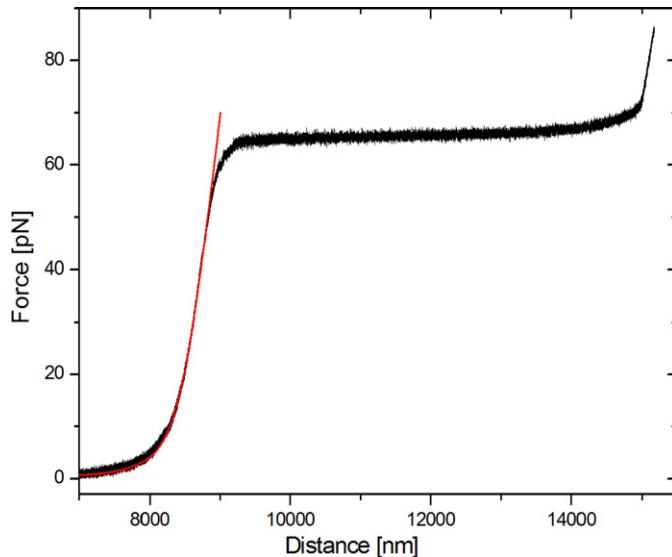


Fig. 4. Single-molecule overstretching of a ~ 24 kbp fraction of lambda DNA. The DNA segment was tethered between a streptavidin coated bead (diameter ~ 2.0 μm) and a bead labelled with anti-DIG (diameter ~ 2.8 μm). The actual data recorded at 1 kHz are shown in black while the fit to the extended WLC (force range of 10–50 pN) yielding a persistence length $L_P = 41 \pm 5$ nm, a contour length $L_C = 8638 \pm 200$ nm and an elastic correction factor $K = 1143 \pm 200$ pN is shown in red. In order to use the extracted parameters for the analysis of single-molecule transcription experiments, experiments were performed in the same buffer (50 mM HEPES pH 7.5, 40 mM $(\text{NH}_4)_2\text{SO}_4$, 10 μM ZnCl_2 , 5% glycerol, 10 mM DTT, with 5 mM MgCl_2). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

When stretching single DNA molecules using single-molecule force spectroscopy one can measure forces already for relatively low extensions (i.e. end-to-end distances) of the DNA molecule due to the conformational flexibility of the DNA [66]. In this regime the molecule behaves like an entropic spring [64]. Upon extension of the molecule the maximum number of its conformational states is reduced. Therefore, in order to extend the molecule, one has to put energy into the system, thus resulting in a measurable force. It has been shown that the shape of the force-extension curve of the DNA molecule can be described accurately with the wormlike chain (WLC) model [64], in which the DNA is treated as a smoothly bendable rod. In addition, for higher forces above ~ 5 pN one starts to observe enthalpic effects caused by small mechanical deformations of the B-DNA structure [67]. However, at least for large DNA molecules of several thousand bases in length, the force-extension behaviour of DNA molecules can be described accurately by introducing a Hookian term [68] resulting in the so-called extensible worm like chain model [68–71]:

$$x = L_C \left(1 - \frac{1}{2} \left(\frac{k_B T}{F L_P} \right)^{1/2} + \frac{F}{K} \right), \quad (1)$$

yielding the contour length L_C , the persistence length L_P and the enthalpic correction factor K for the molecule [18, 26]. Note, that Eq. (1) is an analytical approximation for the true extensible WLC model [72]. Similar experiments have also been done with double stranded RNA molecules [73]. Knowing the characteristic mechanical parameters for describing the force-extension behaviour is a necessary pre-requisite for performing the experiments on molecular motors such as the bacteriophage portal motor protein and the RNA polymerase which will be described in the next section.

Above 50 pN the force-extension curve cannot be described well with the extensible WLC model. In particular at forces of ~ 65 pN one observes a force-plateau. Here, the molecule extends while the force remains (roughly) constant. This so-called over-stretching transition has been described thoroughly both experimentally and theoretically [69,74]. Single-molecule force spectroscopy allows for the detailed analysis of the extension of the DNA during this over-stretching transition, yielding a characteristic length of the over-stretched DNA of about 170% of the contour length of B-DNA [69], as well as a characteristic helical repeat of ~ 37 bp/turn (compared to 10.5 bp/turn for B-DNA) [49]. These physical parameters provide constraints that must be upheld by a structural analysis of the overstretched state [70,75–77]. Moreover, experiments have successfully analysed ionic effects [65], sequence effects [44] as well as pH effects [78,79] on the overstretching transition.

The nature of the over-stretched state the so-called S-DNA is highly disputed. Experiments by Bloomfield and co-workers seem to indicate that over-stretching is merely a manifestation of DNA melting [78,80]. However, if S-DNA were simply melted DNA the nature of a second plateau in the force-extension curves of double stranded DNA at higher forces, observed by AFM force spectroscopy [81] could not be explained. Moreover, recent Monte Carlo sampling of the DNA over-stretching led to the conclusion that the observed experimental data can only be explained if an S-DNA state different than simply a melted state occurs [82]. In fact, from such computations predictions have been made that could help to manifest the existence of S-DNA which still await experimental tests [83].

The B-S transition is not the only phase transition observed during mechanical studies of DNA molecules. By applying positive supercoils to the DNA in experiments where the DNA is torsionally constrained, one can destabilise the DNA and drive it into a different conformation the so-called P-DNA [18,84]. P-DNA has a helicity of ~ 2.6 bp/turn (compared to ~ 10.5 bp/turn for B-DNA) and such a conformation can only be physically possible if the DNA bases are flipped to the outside. Ironically such a conformation was suggested by Linus Pauling [85] only months before Watson and Crick discovered the B-DNA structure [86]. In fact, combining the results of many experiments it is now possible to obtain a complete phase diagram of DNA where instead of pressure and temperature (which are typically constant for biological systems) the important variables are force and torque [18]. At least one of these extreme forms of DNA, the left handed Z-DNA is known to exist in nature [87]. The B-Z transition occurs when DNA is under-wound a process that occurs quite often within the cell, e.g. during transcription by RNA polymerases.

4. Viral DNA packaging

One of the most interesting examples of DNA mechanics, which has triggered a large amount of theoretical models, is the compaction of DNA inside of small compartments due to condensation by accessory proteins or due to the action of a motor protein which exerts a mechanical force to push DNA into a preformed protein compartment. The latter is a central part of the life cycle of a large number of bacteriophage viruses, which package their viral genome into a preformed protein capsid [88]. During packaging energetic penalties have to be overcome arising from entropic, electrostatic and elastic contributions. Thus energy has to be provided in order to package DNA and this energy is liberated by a molecular motor which sits at the apex of the protein capsid and uses the free energy from ATP hydrolysis to power conformational changes which drive the DNA into the capsid. One of the best studied motor systems is the portal packing motor from bacteriophage $\Phi 29$ [89]. The motor consists of three rings which are connected to each other and formed by five (or six) RNA molecules (termed pRNA), five (or six) ATPases called gp16 and the so-called connector (gp10) which is a homododecamer [88]. While the structure of the homododecamer has been solved using crystallographic studies [90], only low resolution cryo-EM structures exist for both the RNA and the gp16 conformation at the apex of bacteriophage $\Phi 29$ proheads [91]. Therefore, only speculative models about the overall mechanism and the structure of the complete motor assembly exist. However, recent single-molecule experiments using single-molecule photo-bleaching [92], single-molecule fluorescence polarisation [51] and in particular optical tweezers [17,32,93–95] have determined many characteristics of the motor system and have thus helped to refine our picture of the molecular mechanism that underlies this powerful molecular motor. Here, we will review only the findings of the optical tweezers measurements.

Packaging is investigated in the same geometry described in the previous section. Antibodies against the capsid protein are used to immobilise the proheads onto beads, and a biotinylated DNA is used to hold the other end. Complexes are either assembled using pre-initiated complexes [17] or in-situ [93]. The portal motor packages the viral DNA in the pre-formed capsid with a remarkable processivity and speed. However, the velocity of the process is changing over time (Fig. 5) an effect that has been attributed to the build up of a restoring force. The motor can package DNA against external forces as high as 65 pN and simplistic models lead to the notion that during this packaging an internal pressure of ~ 50 atm is developed, numbers which can also be obtained using physical models for DNA compaction including entropic penalties, electrostatics and bending contributions [96,97]. Evidence for a pressure on the order of tens of atmospheres also comes from ejection experiments under varying osmotic pressure [98]. Hence, the bacteriophage might act as a loaded spring and upon binding to a receptor on a target cell, the ring-like connector can open and the DNA is injected into the new cell. Since, the measured changes in packaging velocity with filling fraction are quite low for the first half of the genome [93], this process can only account for a partial transfer of the DNA into the new cell. Most likely other molecular motors such as RNA polymerases take over and pull the DNA into the new cell after the initial part of the DNA has been injected. This notion is strengthened by biochemical exper-

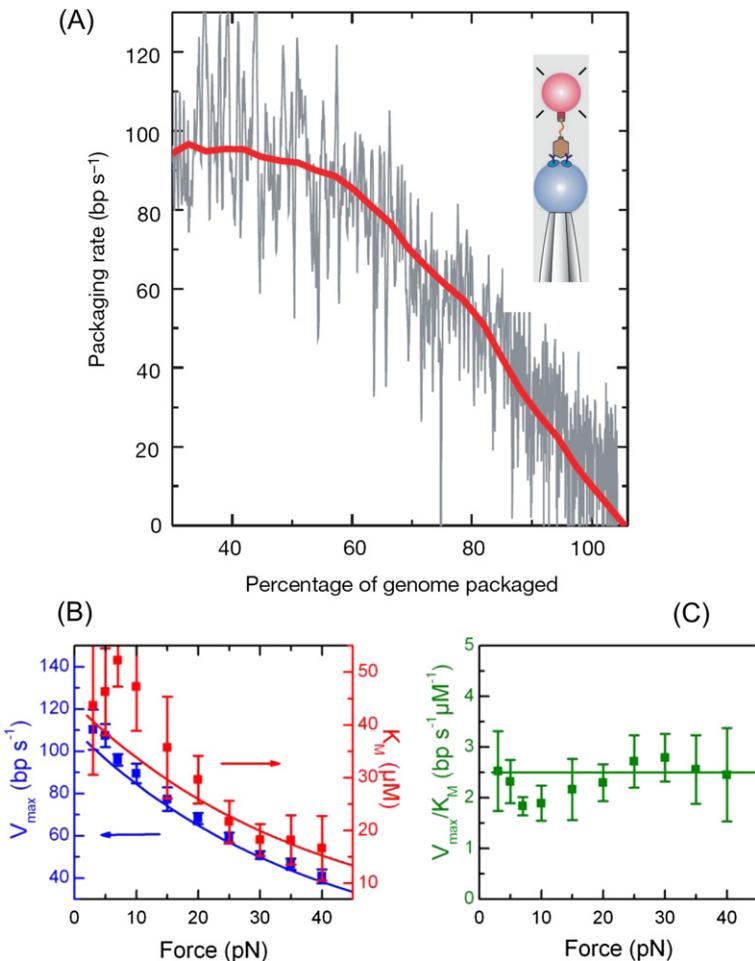


Fig. 5. Viral DNA packaging. Packaging of DNA into a viral capsid by the portal motor complex of bacteriophage $\Phi 29$ was investigated using dual-beam optical tweezers. (A) A schematic of the experimental configuration is shown in the inset. The observed packaging rate varied as a function of the percentage of genome packaged, due to a build-up of an internal opposing force (adapted from Smith et al. [89]). (B) Packaging is slowed down by the applied external load. From experimental data obtained at various ATP concentrations one can calculate the equilibrium constant K_M and the maximum packaging velocity v_{\max} (for saturating ATP) for different applied forces. K_M and v_{\max} both decrease with increasing force. (C) The ratio v_{\max}/K_M is independent of force. Therefore the catalytic rate depends on force and the effective binding rate does not (adapted from Chemla et al. [94]).

iments that tested the infectivity of the virus as a function of the position of a gene encoding for a RNA polymerase along the viral genome [99].

Controlled experiments in optical tweezers also allowed to unravel the mechano-chemistry of the packaging process [94]. It could be shown that albeit several ATPases are assembled into a ring, there is no apparent cooperativity and motion stalls if one ATPase binds to a non-hydrolysable ATP analog. Moreover, the data also show that there is a load-dependence to the catalytic step of the enzyme and that the effective binding itself is force independent (Fig. 5). In addition, force dependent studies of the slippage frequency allowed for the identification of loosely bound states of the motor thus further dissecting the mechano-chemical coupling. However, more direct insight into the molecular mechanism of this fascinating molecular machine comes from recent high-resolution measurements of the packaging process (Fig. 6) [32]. Using two optical traps and a differential detection scheme Moffitt et al. were able to directly resolve individual bursts of activity of the enzyme. Most surprisingly these bursts amounted to fairly large displacements on the order of 10 bp. In their model multiple ATPases in the ring are in a loaded configuration (presumably bound to ATP or the product ADP) and translocation is achieved by a successive conformational change within the ATPases of the ring. Support for this model comes from experiments performed at high loads, where substeps of the

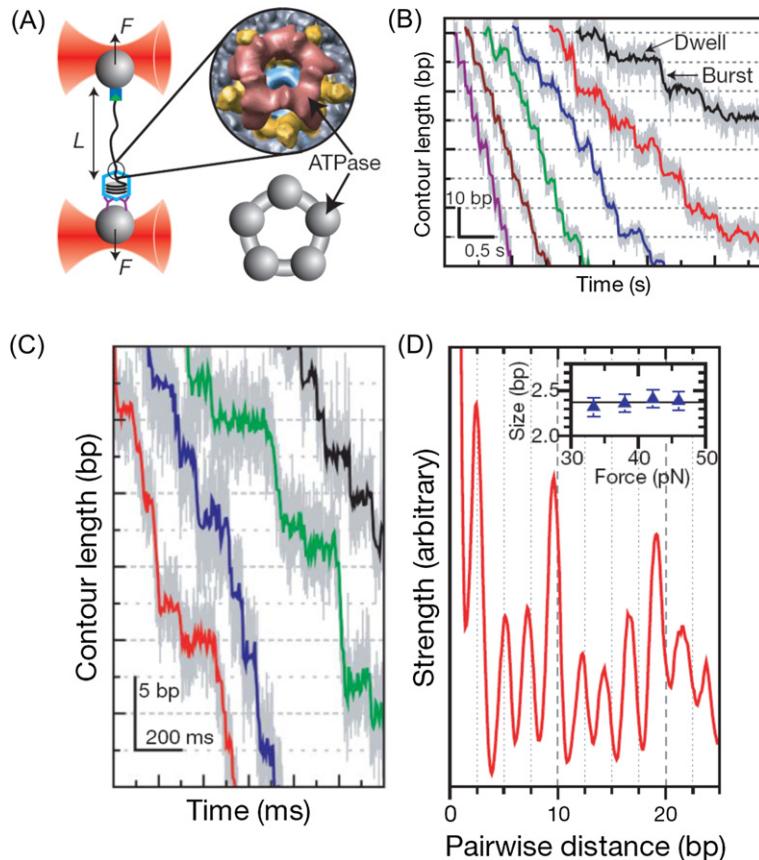


Fig. 6. High-resolution optical tweezers experiments reveal step size of packaging motor (adapted from Moffitt et al. [32]). (A) Schematic of the experimental configuration used for the investigation of DNA packaging in a high-resolution dumb-bell optical tweezers setup. (B) Representative high-resolution packaging data obtained at a force of ~ 8 pN showing distinct translocation dwells followed by packaging bursts of 10 bp in size. Data obtained at 250 (purple), 100 (brown), 50 (green), 25 (blue), 10 (black), 5 μ M ATP (red) are shown. The time between dwells is increased when the ATP concentration is reduced. However, the packaging burst size and dwell-duration is independent of ATP concentration. (C) Representative packaging data obtained at ~ 40 pN and $[ATP] = 250$ μ M. The data shows that at this high load the packaging bursts are broken up by several steps. (D) Pairwise distance analysis of the data obtained at ~ 40 pN. The histogram shows distinct peaks with at 2.5 bp, 5 bp, 7.5 bp, etc. Thus, the bursts are made up of 2.5 bp steps. These steps become apparent at high loads since the high external force increases the waiting time between the 2.5 bp steps. At forces below 30 pN these steps occur so fast, that they cannot be resolved given the limited time-resolution of the instrument. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

10 bp bursts could be observed. Interestingly the size of these substeps was 2.5 bp indicating that either not the full movement of the ATPase is coupled to a DNA movement, or that the movement of the ATPase is independent of the repeat units of the DNA, an effect that has never been observed for an enzyme that actively translocates along DNA. The ability to observe such unexpected step sizes illustrates the power of high-resolution optical trapping techniques and underscores the necessity for developments that improve the accuracy of measurements and characterise systematic errors [32].

5. RNA polymerase

Another important molecular motor that has been investigated intensively over the last years is the (DNA dependent) RNA polymerase. The enzyme has a critical role in gene expression, since it is responsible for making all mRNA in the cell and mRNA production is a highly regulated process. The process of making RNA out of the template DNA molecule is commonly called transcription and is divided into several phases known as transcription initiation, abortive transcription, promoter escape, transcription elongation and transcription termination. Here, we will describe experiments performed using optical tweezers related to the latter two aspects. Conceptually the experimental setup

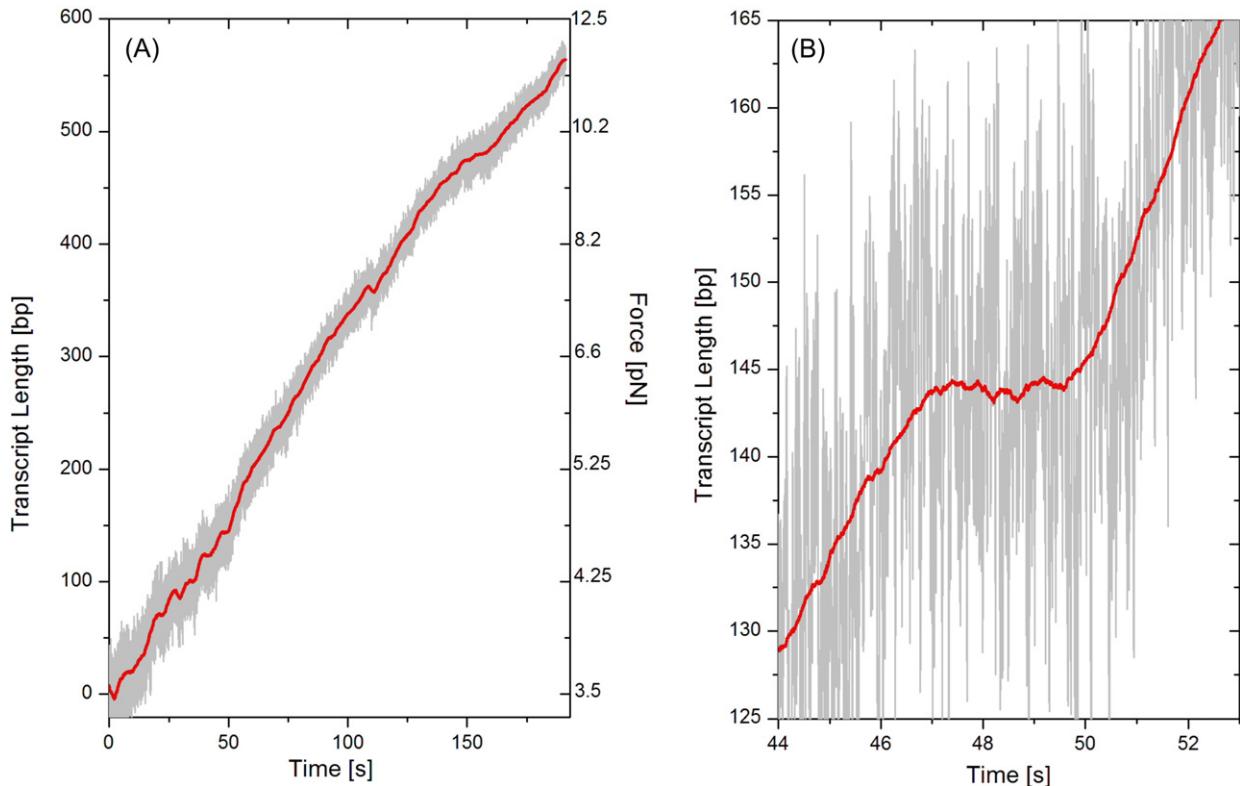


Fig. 7. Single-molecule observation of Pol II transcription. The data were obtained in an opposing force mode depicted in Fig. 3. (A) Representative single-molecule transcription event. The displayed data is filtered at 100 Hz (black) and 0.4 Hz (red) to reduce experimental noise. As the polymerase moves along the DNA, the effective length of the DNA tether is reduced and thus the force is increasing. The right axis shows the recorded force (note, that the force axis is not linear) and the left axis shows the transcribed distance, calculated from the force using the extensible WLC model. The velocity of the transcription is not homogeneous but interrupted by frequent pause. (B) A magnified section of the trace shown in (A) showing the polymerase entering and exiting from a pause. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

is quite similar to the one just described for the investigation of viral DNA packaging. Here, a RNA polymerase is immobilised onto a bead held in an optical trap using antibodies [100] or using a biotinylated enzyme [101]. Most experiments have been performed on the bacterial RNA polymerase (RNAP) but recently experiments were also performed on the eukaryotic RNA polymerase II (Pol II) by the Bustamante lab [102,103] as well as ourselves [100] (Fig. 7). The prokaryotic and eukaryotic enzymes, i.e. the engines of transcription, share a high sequence homology and are structurally virtually identical; however, differences exist in the interaction of the polymerase with co-factors during all phases of transcription. As a general remark, the eukaryotic transcription has evolved to be extremely complex and many accessory proteins (referred to as transcription factors) are used in order to allow for a stringent control of gene expression in the eukaryotic cell.

In order to observe *transcription elongation* of Pol II in an optical tweezer one needs to first assemble a transcription elongation complex. For the eukaryotic enzyme this is quite tricky, since initiation *in-vivo* requires many additional proteins that are so far not available in large amounts at purities high enough to perform single-molecule experiments. However, elongation competent complexes can be assembled *in-vitro* without the need of protein co-factors by using mismatched nucleic acid oligomers [104] or by simply adding first the template DNA–RNA hybrid to the polymerase, before incubation with the nontemplate strand [102,105,106]. In order to fix the DNA-molecular machine complex in between two beads in the optical tweezers, a linker is added to the protein complex by biotinyling a subunit of the polymerase, namely the Rpb4/7 hetero-dimer (or Rpb3 [106]). The elongation complex needs to be ligated to a fairly long DNA molecule which is then used as a tether between the two beads. In analogy to the stretching experiments of bare DNA (see above), inside the chamber a Pol II-DNA tether is formed between two beads. During transcription the polymerase decreases the length of the DNA tether (while increasing the length of the RNA transcript), resulting

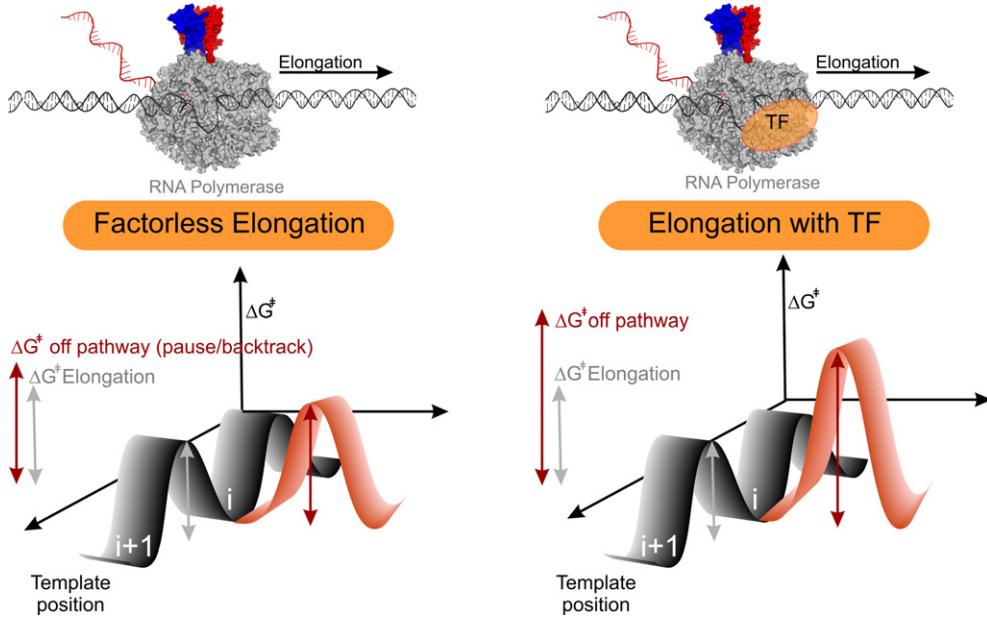


Fig. 8. Influence of transcription factors on transcription elongation. The picture shows a schematic representation describing the function of transcription factors. Transcription elongation can be described by a free energy landscape. During normal elongation the polymerase wanders along the standard elongation pathway (black) and the height of the energy barriers are determined by the particular sequence of the template DNA. Pauses and backtracks can be represented by off pathway events (red). Such an off-pathway state can be reached by crossing a different energy barrier typically higher than the on-pathway barrier. A postulated function of transcription elongation factors that bind to the polymerase (right panel) is to alter the height of the respective energetic barriers for example by increasing the off-pathway barrier. As a result the probability of entering an off-pathway state is reduced and thus the effective elongation rate is increased. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in an increase of force. A typical elongation trace is shown in Fig. 7(A). From the recorded forces, the decrease of the DNA tether length and with that the increase of the RNA transcript length is calculated using the stiffness of the trap, the initial length of DNA and the extensible WLC model.

Transcription elongation is not a homogeneous process, but shows phases where active transcription is interrupted by pauses and backtracks of the polymerase (Fig. 7(B)). Using high-resolution optical tweezers Block and co-workers could show that the polymerase moves along the DNA by stepping one nucleotide at a time [13] using a translocation mechanism that is commonly referred to as a Brownian ratchet. In a Brownian ratchet the enzyme oscillates thermally between two (elongation) states until the final translocated state is locked into place by a local conformational change. In addition to the single-molecule evidence there is also striking biochemical [107] as well as structural evidence [108] in support of the Brownian ratchet model for RNA polymerase translocation. Pauses of transcription occur at specific sequences [109] as well as randomly in time [110,111]. During these pauses the polymerase is prone to backtrack [62] at least if the pause duration is long, however, whether backtracking occurs also for short pauses is currently under dispute [3]. Backtracking can be described by a first passage time problem with an absorbing boundary (the active site). Such a model predicts a distribution of pause times that can be fitted using a $t^{-3/2}$ power law [112]. Interestingly, such a power-law describes the observed pause duration both for the eukaryotic [102] as well as the prokaryotic [112,113] enzyme. However, the issue whether all pauses are accompanied by backtracks is complicated by the fact that only a fraction of actual pauses of the enzyme can be detected experimentally due to limited time and spatial resolution of diffusive backtracking models is that movement through roadblocks (other proteins that are bound to the DNA) which typically appear frequently along the length of the DNA can be facilitated by simply a waiting and ratcheting process. Indication that such a mechanism is actually used by Pol II in order to bypass nucleosomes (the smallest DNA compaction units in the eukaryotic cell) comes from very recent optical tweezers experiments [103]. Bustamante and co-workers demonstrated that the presence of a nucleosome along the DNA drastically increases the propensity of the Pol II enzyme to pause or stall. The pause duration could be reduced by increasing the ion concentration in solution, conditions that are known to weaken the interactions of the histone core proteins to the nucleosomal DNA. Understanding the molecular mechanism how the polymerase can bypass such a roadblock is of

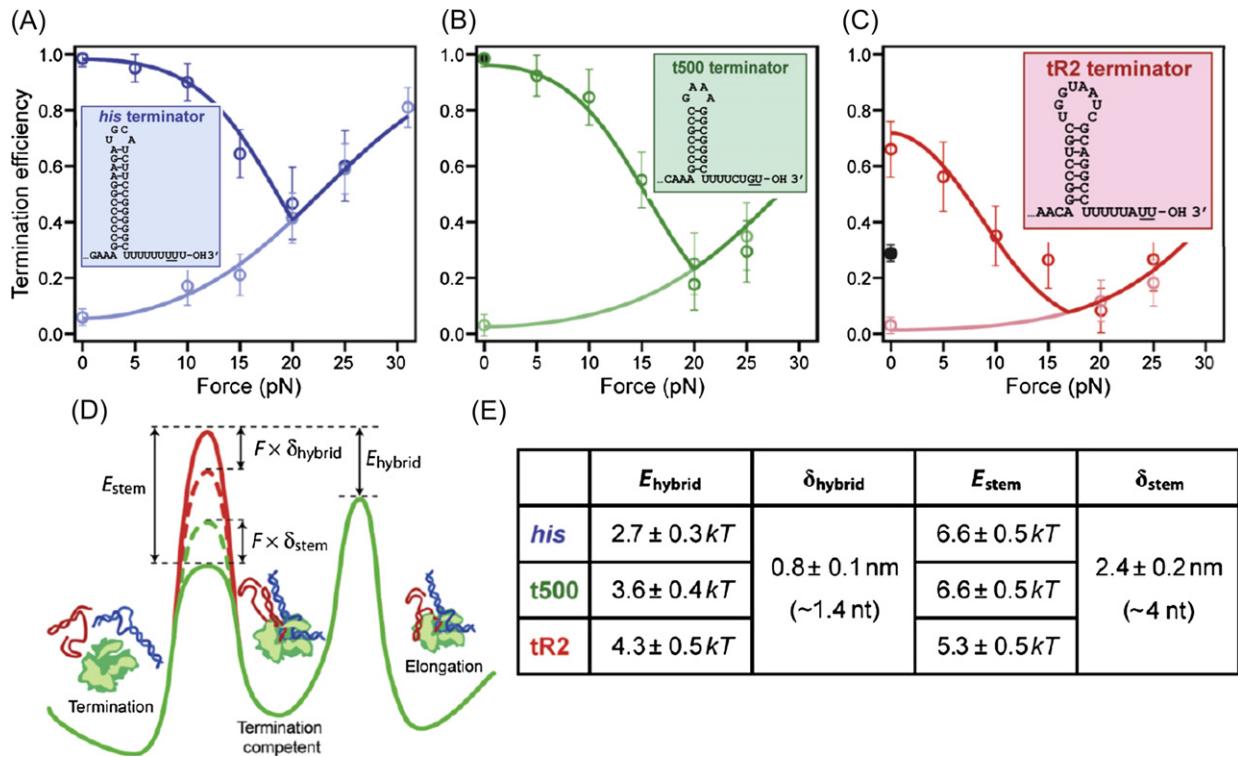


Fig. 9. Sequence dependent transcription termination of the bacterial RNA polymerase. Single-molecule force spectroscopy was applied to determine the termination efficiency of RNAP for different termination sequences. (A)–(C) Termination efficiency for three different termination sequences termed his terminator, t500 terminator and tR2 terminator sequence as a function of applied force. In order to differentiate between contributions to the termination efficiency of the U-tract as well as the hairpin region of the particular terminator sequence, experiments were carried out in presence (dark colour) and absence (light colour) of the hairpin motives. (D) Free energy landscape describing the termination process. From the elongation state a conformational change has to occur to enter the so-called termination competent state. From this state termination occurs by crossing another energy barrier. (E) Table describing the obtained results in terms of the free energy landscape picture. Figure adapted from Larson et al. [114]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

immediate relevance for open questions regarding gene expression. In particular it is interesting to determine how accessory proteins, commonly referred to as transcription factors alter the underlying potential energy surface of the enzyme (Fig. 8). Using force, one can alter this energy landscape and thus determine the barrier height and distance to the transition state for different conditions thus gaining mechanistic insights into the function of transcription factors during transcription elongation.

The same approach was recently used to investigate the mechanism of *transcription termination* for the bacterial RNA polymerase [114]. In prokaryotes a large fraction of transcripts is terminated by so-called intrinsic terminators, i.e. sequences that drastically increase the probability for termination. Block and co-workers studied the termination efficiency of the enzyme at different previously identified termination sequences as a function of force. Termination sequences are known to consist of a GC-rich region which is likely to form a hairpin followed by a U-rich region (oftentimes referred to as a U-tract) that might increase the tendency of the polymerase to slip. Using an applied force to either oppose the polymerase movement, or to assist it by pulling on either the DNA or the nascent RNA transcript they were able to quantify all energetic contributions to termination and developed a complete quantitative model for termination (Fig. 9). In fact, the information gathered is so precise that the authors state that they can (based on their model) quantitatively predict the termination efficiency of any termination sequence, a claim yet to be challenged.

6. Conclusions and outlook

With the accomplishment of the (human) genome project we now have the complete letter code information of DNA. However, in the biological context oftentimes not the sequence information per se, but rather the mechanical

nature of the DNA molecule, that is how easy it can be bend or twisted is important. In particular for proteins that bind to DNA, e.g. nucleosomes or DNA translocating enzymes. We have reviewed important methods to mechanically manipulate DNA such as AFM, magnetic tweezers and in particular optical tweezers and described the features of these techniques, achievements and shortcomings. With these methods one can accurately determine the mechanical properties of DNA. E.g. the observed force-extension behaviour can be described very accurately with an extensible worm like chain model and phase transitions to other stable forms of DNA are observed under high tension or twist. With this knowledge about the mechanical properties of DNA molecules one can turn to the direct investigation of the behaviour of molecular motors that travel along the DNA, such as the eukaryotic RNA polymerase or the viral DNA packaging motor. Therefore, single-molecule manipulation techniques are an important tool that allow for a better understanding of molecular motors and in return a mechanistic understanding of important cellular processes such as transcription, replication [25], translation [115] and many others.

Acknowledgements

We would like to thank Florian Brückner and Patrick Cramer for providing us with Pol II enzyme. This work was funded by the Deutsche Forschungsgemeinschaft SFB 486 and SFB 646 as well as by the Nanosystems Initiative Munich.

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