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MATERIALS AND METHODS.

Protein Cloning and Purification

RecBCD was purified from *E. coli* JM109 co-transformed with plasmids pPB800 and pPB520, which were a generous gift from Dr. Jeff Gelles (Brandeis University) [1,2]. Cells were grown in 2YT in the presence of 34 μg ml^{-1} chloramphenicol and 50 μg ml^{-1} carbenicillin. The cells were grown to an OD_{600} ~0.6, induced with IPTG, collected 4 hours after induction, resuspended in buffer R (50 mM Tris-HCl pH 7.5, 0.1 mM PMSF, 10% sucrose), and lysed by freezing and sonication [1]. The lysate was clarified by high-speed centrifugation and fractionated with 0.282 g ml^{-1} ammonium sulfate. The precipitated protein was recovered by centrifugation at 20,000g for 20 min. The pellet was resuspended in buffer A (20 mM Tris-HCl [pH 7.5], 0.1 mM DTT, 0.1 mM EDTA) and loaded onto a 5 ml HiTrap Q FF column (GE Healthcare). The protein was eluted with a gradient to 100% buffer B (20 mM Tris-HCl [pH 7.5], 0.1 mM DTT, 0.1 mM EDTA, 1 M NaCl). The protein-containing fraction were diluted with buffer A to a final NaCl concentration of less than 0.1 M, and loaded onto a HiPrep Heparin 16/10 FF column (GE Healthcare). The protein was eluted with a gradient to 50% buffer B, loaded directly onto a Mono Q 5/50 GL column, and eluted with a gradient to 100% buffer B. The pooled protein fractions were loaded onto a HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare). Following purification, the protein was dialyzed into storage buffer (50 mM Tris-HCl [pH 7.5], 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol) and stored at -20°C. Care was taken to minimize exposure of the protein to high NaCl concentrations to avoid dissociation of the RecD subunit [3]. Protein concentration was determined using $\varepsilon_{280} = 4 \times 10^5\ M^{-1}\ cm^{-1}$ [3]. An 8% SDS-PAGE gel stained with SafeStain (Invitrogen) indicated that the preparation was >95% pure with a 1:1:1 ratio for the three protein components. Exonuclease and DNA-dependent ATPase activities were assayed for the wild type protein (see below) [1,3,4].

Wild-type EcoRI was purchased from New England Biolabs. Constructs encoding the hydrolytically defective $EcoRI^{E111Q}$ was a generous gift from Dr. Paul Modrich (HHMI & Duke University) [5,6]. For overexpression and purification the gene for $EcoRI^{E111Q}$ was sub-cloned into the pTXB3 vector (New England Biolabs), generating vector pTXBERI. To prepare $EcoRI^{E111Q}$ with a triple FLAG epitope tag repeat, the $EcoRI^{E111Q}$ gene was PCR amplified with oligos IF01 and IF02 (see Table S2). The PCR amplicon was digested with *SapI* and *NcoI* (New England Biolabs) and ligated into pTXB3 to generate plasmid pTXBERI-3FL. The plasmid was transformed into HMS174 DE3 (pLysS) cells. The cells were grown to an OD_{600} ~0.6 in the presence of 34 μg ml^{-1} chloramphenicol and 50 μg ml^{-1} carbenicillin, induced with IPTG,

collected 4 hours after induction, resuspended in buffer R, and lysed by freezing and sonication. The lysate was clarified by high-speed centrifugation and loaded onto a chitin binding domain column according to the manufacturer suggested protocol (New England Biolabs). The column was washed with 20 column volumes of washing buffer W (20 mM Tris-HCl [pH 8.5] and 0.5 M NaCl). The intein-CBD tag was cleaved by flushing the column with buffer W supplemented with 50 mM DTT and incubating overnight at 4˚C. Protein-containing fractions were pooled, dialyzed into storage buffer (40 mM Tris-HCl [pH 7.5], 300 mM NaCl, 10 mM 2-Mercaptoethanol, 0.1 mM EDTA, 50% Glycerol, 0.15% Triton X-100), and stored at -20˚C.

Wild-type RNA polymerase holo- and core-enzymes were purchased from Epicentre Biotechnologies, and were used in bulk experiments as controls. The single molecule experiments used either FLAG tagged RNAP or biotinylated RNAP. RNA polymerase containing an N-terminal 6-His and C-terminal triple FLAG tagged α-subunit were prepared from constructs generously provided by Dr. Karen Adelman (NIEHS) [7]. Plasmid p706a [7] was digested with *BsiWI* and *XhoI* and ligated with oligos IF03 and IF04 (see Table S2). The resulting plasmid, p706a-3FL, was transformed into HMS174 (DE3) pLysS cells. The 6His-tagged RNA polymerase was purified as described previously [7-9]. Prior to separation of the holo- and core enzymes on a MonoQ column [9], an additional HisTrap column was used to separate the FLAG tagged from wild-type proteins [7]. Experiments employing a biotinylated RNA polymerase were purified from a construct generously provided by Dr. Robert Landick (University of Wisconsin-Madison) [10]. Cells harboring a chromosomal copy of the *E. coli* RNA polymerase with an *in vivo* biotynilation peptide on the C-terminus of the β' subunit were purified as described [8,9] and the core and holoenzyme fractions were separated on a MonoQ column [9]. Protein activity for all RNAP constructs was assayed by *in vitro* transcription runoff assays (see below).

Nucleosomes where prepared as described [11]. Histones (H2A, H2B, H3, H4 and 3xFLAG-H3) were expressed in *E. coli*, purified from inclusion bodies and reconstituted as described [12]. In brief, inclusion bodies were resuspended in unfolding buffer (7 M guanidinium-HCl, 1 M NaCl, 50 mM Tris-HCl [pH 7.8], 1 mM EDTA, 1 mM DTT), dialyzed against urea buffer (7 M urea, 10 mM Tris-HCl [pH 7.8], 1 mM EDTA, 5 mM β-mercaptoethanol and 100 mM NaCl for H2A, H2B, 3xFLAG-H3 or 200 mM NaCl for H3, H4), then loaded onto tandem HiTrap Q and SP columns (GE Healthcare). 3xFLAG-H2B was expressed in *E. coli*, precipitated from cell lysate in 50% NH_3SO_4, dialyzed against urea buffer and 100 mM NaCl and loaded onto HiTrap SP column (GE Healthcare). Histones were eluted from the SP column with a 100-400 mM NaCl gradient for H2A, H2B, and 3xFLAG-H2B and 3xFLAG-H3 and a 200-500 mM NaCl gradient for H3 and H4. Purified histones were dialyzed against 10 mM Tris-HCl [pH 7.8] plus 5

mM β-mercaptoethanol, followed by 10 mM Tris-HCl [pH 7.8], then lyophilized and stored at –20˚C. Lyophilized histones were unfolded in 7 M guanidinium-HCl, 50 mM Tris-HCl [pH 7.8] plus 10 mM DTT, combined at equimolar ratios, and dialyzed into 2 M NaCl, 20 mM Tris-HCl [pH 7.8], 1 mM EDTA, 5 mM β-mercaptoethanol with several buffer changes over 48 hours. Reconstituted octamers were purified by gel filtration and deposited onto DNA by salt dialysis [12,13].

Wild-type LacI was PCR amplified for plasmid pTYB21 (New England Bioloabs) using oligos IF11 and IF12 and sub-cloned into pTXB3, generating pTXLACI. A $FLAG_6$ epitope tag was introduced at the C-terminus by ligating oligos encoding FLAG repeats at the C-terminal *ClaI* restriction site to generated pTXLACI-6FL. Plasmids encoding either *wt* or FLAG-labeled LacI were transformed into BL21 (DE3) cells, grown to OD_{600}~0.6 in the presence of 50 μg ml^{-1} carbenicillin, and induced with 2.5 mM IPTG. After a three-hour induction, the cells were harvested and purified using the same protocol as for EcoRI (see above).

Bulk Characterization of RecBCD

RecBCD activity was verified using ATPase reactions under the same buffer conditions as the single-molecule flowcell assay. Reaction mixtures containing 1.5 nM (in molecules; 3nM in DNA ends) λ-DNA (New England Biolabs), 0.1 μM [α-^{32}P]ATP, 1 mM ATP, 40 mM Tris-HCl [pH 8], 2 mM $MgCl_2$, 0.2 μg μl^{-1} BSA, and 1 mM DTT. For the time courses, reactions contained 0.1 nM RecBCD and were terminated with the addition of EDTA to a final concentration of 80 mM after incubations for the indicated periods (Supplemental Fig. S1). Reaction products were resolved by thin layer chromatography on PEI-cellulose (Sigma Aldrich) run in 0.5 M formic acid with 0.5 M LiCl. Products were detected with a PhosphorImager, and analysis was done with ImageQuant 5.2 software.

Exonuclease assays were carried out essentially as described previously [14], and confirmed the proteins were fully active (not shown). Under reaction conditions were [ATP] << [Mg^{2+}], RecBCD nuclease activity produces short oligonucleotide products that can be separated from intact duplex DNA by trichloroacetic (TCA) acid precipitation [14,15]. Exonuclease activity was followed by observing the time dependent release of radio-labeled oligonucleotides that remain soluble after TCA precipitation. Substrate DNA was produced by PCR amplifying a 965 bp region of λ-DNA (primers: 5'-GCTGGCTGACATTTTCGGTGC-3' and 5'-GCCACGCCCATTAGTGAAACG-3') using *Pfu* DNA polymerase (Stratagene) as described by the manufacturer with the addition of 1 μCi [α-^{32}P]dATP. The PCR product was purified with a Qiagen MinElute kit, and the concentration quantified by UV-VIS, assuming one OD_{260} unit is 50

μg ml^{-1} for dsDNA. The specific activity of the dsDNA was determined to be 356,000 cpm $nmol^{-1}$ using liquid scintillation counting.

Reactions containing 50 mM Tris HCl [pH 8.5], 10 mM $MgCl_2$, 40 μM ATP, 40 μM dsDNA (in nucleotides), 0.67 mM DTT, and 0.2 μg μl^{-1} BSA were pre-incubated at 37°C for several minutes. Reactions were initiated by addition of RecBCD (in reaction buffer minus DNA and ATP) to a final concentration of 0.1 nM. At the indicated time points, 20 μL aliquots were withdrawn and quenched by adding 100 μL of 10% ice-cold TCA and 5 μL of 0.5 mg ml^{-1} sheared salmon sperm DNA (Ambion). The quenched aliquots were kept on ice for at least ten minutes, and undigested DNA collected as an invisible pellet by centrifugation at ~14,000 rcf for 10 minutes at 4°C. Following centrifugation, 110 μL of the supernatant was added to 5 mL of scintillation counting fluid (Fisher Scientific) and quantified by liquid scintillation counting. All measurements were performed at least three times. Control experiments containing all components except ATP indicated no nuclease activity (data not shown).

Bulk Characterization of RNA Polymerase

RNA polymerase run-off transcription assays were performed to ensure that the introduction of an epitope tag and QD labeling did not adversely affect enzymatic activity (Supplemental Figure S2). A 467 bp segment of λ-DNA containing the λP_L promoter was amplified by PCR using cloned *Pfu* DNA polymerase (Stratagene) and primers IF09 and IF10. The amplicon was purified with a QIAGEN PCR purification kit [16]. Transcription assays were carried out in 25 μl reactions containing transcription buffer (40 mM Tris-HCl [pH 8.0], 150 mM KCl, 10 mM $MgCl_2$, 250 μM each rNTP) supplemented with 0.5 μCi [α-^{32}P]ATP, 40 U SUPERase•In RNAse inhibitor (Ambion), 40 nM dsDNA template, and 20 nM holo-RNA polymerase. When indicated, QDs were added to a final concentration of 40 nM. The reaction was incubated for 1 hr at 37°C. Reactions were centrifuged through a NucAway desalting column (Ambion) and mixed with 2x RNA loading dye solution (Fermentas). Transcription products were resolved on 6% polyacrylamide-urea gels (Invitrogen). Gels were run at room temperature for 1 hr (constant voltage, 175V) in 1x TBE running buffer. The expected transcription products [16] were detected with a Typhoon PhosphorImager (GE), and analysis was done with ImageQuant 5.2 software.

Single-turnover transcription experiments were performed on stalled RNAP elongation complexes to determine the percent of stalled polymerases that can re-initiate transcription [17]. Stalled complexes were prepared by incubating 20 nM holo RNAP with 40 nM dsDNA template in transcription buffer supplemented with 40 U SUPERase•In RNAse inhibitor, 150 μM ApU

(RiboMed), 0.5 μCi [α-^{32}P]ATP and 25 μM each of rATP, rCTP, rGTP. The reaction was incubated for ten minutes at 37˚C. Half of the reaction was quenched with EDTA to 25 mM and kept on ice. The second half-reaction was rapidly supplemented with 0.1 mg/ml heparin and 250 μM each of rATP, rCTP, rGTP, rUTP. After a five minute incubation at 37˚C, transcription products were resolved on an 18% polyacrylamide-urea gel (Invitrogen). Gels were run at room temperature for 1:45 hr (constant voltage, 175V) in 1x TBE running buffer and analyzed as above. All transcription reactions were repeated at least three times.

Bulk Characterization of EcoRI^{E111Q}

EcoRI digestion protection assays were performed essentially as described [18]. λ-DNA (1.5 nM; five EcoRI sites) was incubated with 30 nM EcoRI^{E111Q} (concentration in dimers) in binding buffer (25 mM Tris-Cl [pH 8.0], 150 mM NaCl, 1 mM DTT, 0.2 μg μl^{-1} BSA, 10 mM $MgCl_2$) for 30 minutes on ice, followed by 2 minutes at 37˚C. Where indicated, QDots were included as a two-fold excess over EcoRI^{E111Q} dimers. DNA-EcoRI^{E111Q} complexes were challenged with 10 U μl^{-1} *wt*EcoRI (New England Biolabs) for 5-60 minutes at 37˚C. Reactions were stopped with 20 mM EDTA, 0.5% SDS and deproteinized with 1 mg ml^{-1} Proteinase K for 30 minutes at 50˚C. The products were run at 80V on a 0.6%, room temperature agarose gel, stained with ethidium bromide, and imaged on a UV trans-illuminator (SynGene). Time-courses (data not shown) were in agreement with previously published results [18]. All assays were repeated at least three times.

DNA Substrates for Single Molecule Experiments

Most experiments used bacteriophage λ (48,502 bp) lacking a Chi-sequence. To determine whether RecBCD could strip obstacles when RecB was the lead motor a triple Chi-sequence [19] was cloned into bacteriophage λ. *In vitro,* RecBCD recognizes each Chi-sequence with a 30% probability [20] and a triple Chi-sequence repeat was previously shown to be recognized by 90% of translocating RecBCD molecules [19]. Phage DNA harboring the *cI857* and *S7* mutations (New England Biolabs) was digested with *XhoI* and *NheI* and ligated with oligos IF05 and IF06 (Table S2), which abolish both cut sites. The ligated product was digested again with *XhoI* and *NheI* prior to phage DNA packaging according to the manufacturer supplied protocol (EpiCentre). Individual phage plaques were screened for the insert by PCR and confirmed by DNA sequencing. Phage particles were isolated from heat-inducible *E.coli* lysogens and the DNA containing the Chi locus was purified with a commercial phage DNA extraction kit (Qiagen). The final DNA construct containing Chi was 47,321 bp.

For experiments with LacI-RecBCD collisions, a single high-affinity "ideal" LacO sequence [21] was introduced into λ-phage by ligating the complimentary oligonucleotides IF13 and IF14 between *XhoI* and *NheI* restriction sites, as described above. The final DNA construct containing LacO was 47,331 bp.

Phage DNA with a biotinylated *cos* end was prepared according to previously published protocols [22]. Briefly, the *cos* end was annealed with oligos IF07 or IF08 (Table S2), ligated and filtered over an Sephacryl S-200 HR column (GE Healthcare) to remove excess oligonucleotide and ligation reaction components.

Quantum Dot Preparation

Streptavidin-quantum dots (QDs) were purchased from Invitrogen. To prepare antibody labeled QDs, amine-functionalized QDs were labeled with affinity purified, reduced anti-FLAG antibodies (Sigma-Aldrich) using SMCC (succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate). The resulting QD-antibody conjugates were then purified over a Superdex 200 10/300 GL gel filtration column (GE Healthcare), and were stored in PBS [pH 7.4] at 4°C. Although the manufacturer-reported antibody:QD ratio is ~4:1, the percentage of QDs with an active antibody may be substantially lower [23]. There may be "dark" proteins that are not coupled to QDs or QDs that are not fluorescent [24]. The presence of dark QDs or unlabeled proteins would increase the total number of RecBCD-roadblock collisions, but those involving dark roadblocks would not be observed. The possible presence of dark roadblocks does not affect any of our conclusions.

Nanofabrication, Flowcells, Lipid Bilayers, and DNA Curtains

A complete description of the DNA curtains made with nanofabricated diffusion barriers containing geometric nanowells can be found in Visnapuu *et al.*, 2008 [25]. In brief, fused silica slides (G. Finkenbeiner, Inc.) were cleaned in NanoStrip solution (CyanTek Corp, Fremont, CA) for 20 minutes, rinsed with acetone and isopropanol and dried with N_2. Slides were spin-coated with a bilayer of polymethylmethacrylate (PMMA; 25K and 495K; MicroChem, Newton, MA), followed by a layer of Aquasave (Mitsubishi Rayon). Patterns were written with a FEI Sirion scanning electron microscope (J. C. Nabity, Inc., Bozeman, MT). Aquasave was removed with deionized water and resist was developed using isopropanol:methyl isobutyl ketone (3:1) for 1 minute with ultrasonic agitation at 5°C. The substrate was rinsed in isopropanol and dried with N_2. Barriers were made with a 15-20 nm layer of chromium (Cr), and following liftoff, samples were rinsed with acetone and dried with N_2, as described [25].

Inlet and outlet ports were made by boring through the slide with a precision drill press equipped with a diamond-tipped bit (1.4 mm O.D.; Kassoy). The slides were cleaned by successive immersion in 2% (v/v) Hellmanex, 1 M NaOH, and 100% MeOH. Slides were rinsed with MilliQ™ between each wash and stored in 100% MeOH until use. Prior to assembly, slides were dried under a stream of nitrogen and baked in a vacuum oven for at least 1 hour. A sample chamber was prepared from a borosilicate glass coverslip (Fisher Scientific) and double-sided tape (~100 μm thick, 3M). Ports (Upchurch Scientific) were attached with hot-melt adhesive (SureBonder glue sticks, FPC Corp.). The total volume of the sample chambers was ~14 μl. A syringe pump (Kd Scientific) and actuated injection valves (Upchurch Scientific) were used to control sample delivery. The flowcell and prism were mounted in a custom-built heater with computer-controlled feedback regulation.

Lipids were purchased from Avanti Polar Lipids and liposomes were prepared as previously described [25]. In brief, a mixture of DOPC (1,2-dioleoyl-*sn*-glycero-phosphocholine), 0.5% biotinylated-DPPE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl)), and 8% mPEG 2000-DOPE (1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]). The mPEG prevented nonspecific adsorption of QDs. Liposomes were applied to the sample chamber for 30 minutes. Excess liposomes were removed with buffer containing 10 mM Tris-HCl [pH 7.8] and 100 mM NaCl. The sample chamber was then flushed with buffer A (40 mM Tris-HCl [pH 7.8], 1 mM DTT, and 1 mM $MgCl_2$) plus 0.2 mg ml^{-1} BSA for 5 minutes. Streptavidin (0.02 mg ml^{-1}) in buffer A was injected into the sample chamber and incubated for 20 minutes. After rinsing with additional buffer A plus 0.2 mg ml^{-1} BSA, λ-DNA (15-20 pM) labeled at one end with biotin and pre-stained with 0.5 nM YOYO1 was injected into the chamber, incubated for 10 minutes, and unbound DNA was removed by flushing with buffer at 0.1 ml min^{-1}. Application of flow aligned the DNA molecules along the diffusion barriers, pushed them into the nanowells, and stretched the molecules parallel to the sample chamber surface. We could readily distinguish nanowells harboring 1 DNA from those containing 2 or more molecules based on YOYO1 signal intensity [25], and RecBCD translocation data were only collected from nanowells containing single full-length DNA molecules (see below).

Single Molecule Assays

DNA curtains without any roadblock proteins were assembled on nanofabricated silica slides as described above. RecBCD was diluted to 20 nM in imaging (IM) buffer (40 mM Tris-

HCl [pH 8.0], 2 mM $MgCl_2$, 1 mM DTT, 0.5 nM YOYO1 (Invitrogen), 50 mM β-mercaptoethanol, 1.4 mM glucose, glucose oxidase and catalase)[26], and injected slowly (70 μl min^{-1}) into the flowcell over the course of several minutes. After excess RecBCD was washed out, the flow rate was increased to 400 μl min^{-1}, and data acquisition was initiated. At this flow rate, the DNA was approximately 80% extended relative to its full contour length (see below). A hundred frames were acquired prior to switching to digestion buffer (IM buffer supplemented with 1 mM ATP, unless otherwise indicated). Upon ATP injection, RecBCD begins to unwind and nucleolytically degrade dsDNA, leading to a reduction in DNA length and concomitant ejection of YOYO1, and the time-dependent DNA length is used as a readout of RecBCD translocation (see below) [27,28]. All experiments were conducted at 37˚C.

DNA curtains with stationary holo- or core-RNA polymerase enzymes were prepared using biotinylated RNAP. The biotinylated polymerase was incubated with a five-fold molar excess of Streptavidin-conjugated QDs on ice for ~15 minutes. After labeling, the reaction was diluted to 1 nM RNA polymerase (5 nM QDs) with RNAP Buffer (40 mM Tris [pH 8.0], 100 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.2 mg ml^{-1} BSA) supplemented with ~10 μM free biotin. Prior to QD-RNAP injection, remaining biotin-binding sites on the flowcell surface-immobilized streptavidin were blocked by incubating the flowcell in imaging buffer containing ~10 μM free biotin. Control experiments indicated that all free flowcell streptavidin sites were blocked by biotin. QD-RNA polymerase was injected into the flowcell at 70 μl min^{-1} in RNAP buffer over the course of several minutes. Inclusion of free biotin in the flow buffer further prevented potential interactions between the biotinylated enzyme and lipid-bound streptavidin. After all free RNA polymerase and biotin were flushed out of the sample chamber, flow was switched to IM buffer for further RecBCD experiments. Reactions with FLAG-tagged RNAP were carried out by pre-incubating 20 μl of ~150 pM λ-DNA with 1 μl of 100 nM FLAG-RNAP in RNAP buffer for 10 minutes at 37˚C. The reaction was diluted to a total volume of 1 ml with RNAP buffer, injected into the flowcell, and incubated 5 minutes at room temperature, allowing the biotinylated ends to attach to the streptavidin on the lipid bilayer surface. Excess DNA and RNAP were flushed out and the FLAG-tagged proteins were labeled *in situ* by flushing 700 μl of 1 nM anti-FLAG QDs through the flowcell in IM buffer. The use of FLAG-tagged RNAP eliminated the need to block the sample chamber surface with free biotin.

Experiments involving transcribing RNA polymerase molecules were carried out using the FLAG-tagged construct. Stalled elongation complexes were prepared on λ–DNA as described [7,8]. Briefly, holo-RNA polymerase was diluted to 15 nM in 20 μl of Buffer T (10 mM Tris [pH 8.0], 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT) supplemented with 150 pM λ–DNA,

150 μM ApU, 25 μM rATP, 25 μM rGTP, and 25 μM rCTP for 10 minutes. After incubation, the reaction was diluted to a total volume of 1 ml with RNAP buffer supplemented with 0.2 mg ml^{-1} heparin, injected into the flowcell, and incubated 5 minutes at room temperature. Heparin effectively competes for DNA binding, dissociating all RNAP complexes that are bound at nonspecific λ–DNA sites (see Figure S2) [29]. Free proteins and rNTPs were washed out and the DNA-bound RNA polymerases were labeled *in situ* with anti-FLAG QDs as described above. Active elongation complexes were prepared from stalled ECs by injecting buffer T supplemented with 250 μM CTP, GTP, UTP, and 1 mM ATP (to maintain consistent RecBCD velocity).

For DNA curtain assays using $EcoRI^{E111Q}$, 20 μl of ~150 pM λ-DNA (750 pM EcoRI sites) was incubated with 1 μl of 100 nM $EcoRI^{E111Q}$ (concentration in dimers) in EcoRI Buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10 mM $MgCl_2$, 0.2 mg ml^{-1} BSA) for 30 minutes on ice, followed by 5 minutes at 37˚C. The reaction was diluted to a total volume of 1 ml with EcoRI buffer, injected into the flowcell, and incubated 5 minutes at room temperature allowing the biotinylated DNA ends to attach to the streptavidin on the lipid bilayer surface. Excess DNA and $EcoRI^{E111Q}$ were flushed out and the FLAG tagged proteins were labeled *in situ* as described above.

DNA curtains with specifically-bound LacI were constructed as for $EcoRI^{E111Q}$ above. λ-DNA containing LacO (20 μl of ~150 pM DNA) was incubated with 1 μl of 50 nM LacI (concentration in dimers) in LacI Buffer (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 0.2 mg ml^{-1} BSA) for 5 minutes at 37˚C. The reaction was diluted to a total volume of 1 ml with LacI buffer. After the DNA curtain was constructued and excess protein and DNA flushed out of the flowcell, LacI was labeled with antiFLAG QDs *in situ*.

Control experiments with LacI incubated with *wild type* λ-DNA (lacking a LacO sequence) indicated that the enzyme bound the non-specific DNA transiently and rapidly dissociated from the DNA in the presence of buffer flow. Lifetimes of non-specific LacI-DNA complexes were estimated by incubating FLAG-LacI with a five-fold excess of antiFlag-QDs and injecting the enzyme into flow-cells with pre-formed *wild type* λ-DNA curtains. The reaction buffer, flow rate and illumination conditions were identical to those used for RecBCD digest experiments. Under these conditions, we observed a number of transient and DNA-specific LacI binding events (N=445) that lasted 0.2-5 seconds. A histogram of 445 LacI-DNA association events (data not shown) was fit with an exponential lifetime of 0.24 seconds, but since the majority of observed events lasted one frame (0.2 seconds), we report an upper bound of 300 msec for the non-specific half-life.

Nucleosome-bound DNA curtains were constructed as described previously [11] and the nucleosomes were labeled *in situ* by flowing 700 μl of 2 nM antiFLAG QDs through the flowcell in IM buffer. Nucleosome collision experiments were conducted as described above for RNAP EcoRI^{E111Q} and LacI.

Single Molecule Data Collection and Analysis

The custom-built total internal reflection microscope used in this study has been described previously [25,30]. Streams of 1000-3000 images were acquired at 5.0 Hz using a 200-millisecond integration time. For RecBCD experiments at lower ATP concentrations and roadblock dissociation measurements, the laser beam was shuttered for up to two seconds between individual 200 ms exposures. All data were collected using NIS-Elements software (Nikon) and saved as uncompressed, 16-bit TIFF files. A Dual-View image-splitting device (Optical Insights) with a dichroic mirror (630 DCXR, Chroma Technologies) was used for two-color detection. This set-up allowed us to simultaneously image the λ_{em}=705 nm QD-protein complexes and YOYO-stained DNA (λ_{em}=509 nm) on separate halves of the same CCD chip. Alignment of red and green channels was performed during post-processing (ImageJ software with "Align RGB Planes" plug-in) using dark signal from the nanofabricated barriers as a reference. Alignment was confirmed by observing fluorescent signals from surface imperfections that were occasionally observed in both channels. Aligned images were pseudo-colored and digitally recombined in ImageJ.

DNA length tracking for experiments involving RecBCD collisions with RNAP or EcoRI^{E111Q} were performed using an automated algorithm (MATLAB) that iteratively fit the intensity profiles of individual YOYO-stained DNA molecules according to equation (1):

$$I(x) = \frac{A}{2}\mathrm{erfc}\left(\frac{-(x_C - x)}{w}\right) + I_0 \qquad (1)$$

where $I(x)$ is the intensity of the YOYO1 signal at pixel x, A is the amplitude, I_0 is the intensity offset, x_c is the center, and w the width of the complimentary error function defined by:

$$\mathrm{erfc}(x) = \frac{2}{\sqrt{\pi}}\int_x^{\infty} e^{-t^2}\,dt$$

To improve signal-to-noise, the intensity profile for each DNA molecule, $I(x)$, was computed by averaging the fluorescent signal from a fifty pixel long and two pixel wide region-of-interest that captured the most intense YOYO fluorescence. The algorithm described by equation (1) tracks the position of the free DNA end, as the surface-tethered DNA end is stationary at the diffusion

barrier. The total length of the DNA molecule was defined as the distance between the tethered DNA end, x_T, and the free DNA end, $x_F = x_c + w$.

In experiments studying RecBCD collisions with nucleosomes, DNA length tracking was performed using a custom algorithm (IgorPro) that iteratively fit the images of individual YOYO-stained DNA molecules according to the equation (2):

$$I(x,y) = I_0 + A \frac{e^{\left(\frac{-(x-x_c)^2}{w^2}\right)}}{\left(1+e^{\frac{(y_{min}-y)}{w}}\right)\left(1+e^{\frac{(y-y_{max})}{w}}\right)} \qquad (2)$$

Where $I(x,y)$ is the YOYO1 intensity at pixel position (x,y). A is the amplitude, I_0 is the background intensity, x_c is the center of the DNA, y_{min} and y_{max} are the top and bottom edges of the DNA, and w is the width of the point spread function.

For both cases, the conversion factor from pixels to base pairs for each flowcell was obtained by averaging the observed length of at least five full-length, undigested DNA molecules and dividing by the known number of base pairs. This conversion factor was computed for every flowcell prior to injection of RecBCD under the same imaging conditions. We restricted data analysis to individual DNA molecules that are full length at the start of the experiment, and are clearly separated from neighboring DNA. The resolution of the DNA tracking algorithm was estimated by tracking several hundred frames of full-length λ-DNA molecules under the same conditions as used for single molecule RecBCD digestion experiments. The fluctuations in the measured DNA length set a resolution of ~1,000 bp for full-length λ-DNA.

QD-tagged proteins were tracked as described previously using an automated algorithm (MATLAB or IgorPro) [11,22]. Briefly, the x and y positions of the point-spread function were fit to a 2D-Gaussian function in conjunction with a region-of-interest mask. Both the x-coordinates (i.e. perpendicular to the long axis of the DNA) and y-coordinates (*i.e.* parallel to the long axis of the DNA molecules) were recorded for each tracked particle. All RecBCD velocity histograms were fit to a Gaussian function using Origin Pro (OriginLabs, Inc.) and the reported values for the means and standard deviations were determined directly from the Gaussian fits to the data. Roadblock dissociation histograms were fit to a single exponential decay in Origin Pro and the reported values are for the half-life and standard fit error.

For all initial experiments, and for all kymograms shown in the manuscript, YOYO1 was used to stain the DNA and locate the curtains. YOYO1 did not affect the translocation rate or processivity of RecBCD, and did not affect the binding distributions of RNAP, EcoRI^{E111Q}, or

nucleosomes (not shown). In the presence of YOYO1 the roadblocks showed the same general response to collisions with RecBCD as seen in the absence of YOYO1. However, when YOYO1 was present the overall distance that the obstacles were pushed was reduced compared to minus YOYO1 reactions, yielding values of 6720±4800, 6670±4390, and 6265±3555 base pairs for RNAP, EcoRI^{E111Q}, and nucleosomes respectively. The reduction in sliding distance, and the similarity of the three values suggests that the YOYO1 was inducing DNA damage (possible nicks) or otherwise perturbing the nucleoprotein complexes, and that the distances over which the obstacles were pushed were limited by the presence of the YOYO1 dye. Therefore all sliding distances and half-lives reported in the main manuscript correspond to values that were measured in the absence of any YOYO1.

Sliding distances were only reported for QD-tagged roadblocks that were pushed along the DNA and did not encounter any other QD-tagged proteins as they moved along the DNA. This ensures that each analyzed collision/dissociation event only involved a single QD-tagged protein. Many reactions were observed in which multiple QD-tagged roadblocks were pushed into one another, but in these cases we could not definitely determine the order in which each different QD-protein was eventually displaced from the DNA, and therefore could not measure sliding distances. Finally, for categorizing the event type distributions we defined "sliding" as any QD-tagged roadblock that moved more than 0.53 μm (approximately 1,950 bp), and anything less than this was scored as a direct dissociation event.

Polystyrene Bead and Alexa Fluor Dye Experiments

Streptavidin-labeled 40-nm far-red fluorescent TransFluoSpheres (488/645) were purchased from Invitrogen (Cat. No. T-10711). To prepare antibody labeled microspheres, 30 µL of 66 nM biotinylated monoclonal anti-FLAG antibodies (Sigma-Aldrich) in conjugation buffer (40 mM Tris-HCl [pH 8.0], 1 mM $MgCl_2$, 1 mM DTT, 0.2 mg/ml BSA) were incubated with 30 µL of TransFluoSpheres at stock concentration (0.5% solids) for 20 minutes on ice. The microsphere-antibody complexes were isolated from free antibodies by centrifugation at 16,100 RCF for 20 minutes at 4˚C. The flowcell surface was blocked with 0.1x saturated biotin solution (C_f≈0.1mM biotin) in conjugation buffer. The pelleted microsphere-anti-FLAG conjugates were diluted to 100 µl of IM buffer and injected into flowcells that contained DNA curtains with pre-bound roadblock proteins as described for the QD labeling experiments. For experiments with biotinylated RNA polymerase, the bead-enzyme complexes was prepared as described above and injected into flow-cells with pre-formed DNA at a slow rate (0.05 ml/min) to facilitate binding of RNAP to the DNA curtain.

The Alexa 488 dye-labeled anti-FLAG antibodies were prepared according to the manufacturer's protocol (Invitrogen; Cat. No. A-20181). In brief, amine-reactive Alexa-Fluor 488 was reacted with monoclonal anti-FLAG antibody (Sigma-Aldrich) and purifed on a size exclusion spin column. Labeling efficiency was calculated according to the manufacture's recommendation: Moles dye per mole protein = A494 / (71,000 × (M)), where 71,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of the Alexa Fluor 488 dye at 494 nm, and (M) = [A280 - (A494 × 0.11)] / 203,000, where 203,000 $cm^{-1}M^{-1}$ is the molar extinction coefficient of the antibody at 280 nm and 0.11 is a correction factor that accounts for the fluorophore's contribution to the absorbance at 280 nm. This calculation yielded a value of ~5 Alexa Fluor 488 dyes per antibody. The fluorescent antibodies were diluted to 5 nM in 1 ml of IM buffer and injected into the flowcell that contained DNA curtains with pre-bound roadblock proteins as described for the QD experiments. Single molecule RecBCD collision experiments were conducted with the bead- and Alexa-labeled roadblock proteins as described above for the QD-labeled roadblock proteins.

Figure S1. RecBCD Translocation on DNA Curtains. (**A**) Schematic of experimental set-up. Bacteriophage λ-DNA (48,502 bp) was anchored by one end to a lipid bilayer through a biotin-streptavidin linkage and aligned within nanowells along the leading edge of a nanofabricated barrier to lipid diffusion through the application of hydrodynamic force. The distance between each nanowell was either 0.5-μm or 1.0-μm, which corresponds to the minimal separation distance between the adjacent DNA molecules within the curtain. RecBCD was loaded at the free DNA ends, excess protein flushed out, and translocation initiated by addition of 1 mM ATP (unless otherwise indicated). YOYO1 is ejected as the dsDNA is unwound and degraded, and the time-dependent decrease in DNA length provides a readout for RecBCD translocation. (**B**) Representative example of a YOYO1-stained DNA curtain (with 1.0-μm nanowell spacing; top), examples of kymograms showing RecBCD translocation on λ-DNA with and without Chi (χ), as indicated (left panels), and representative tracking data that was used to determine translocation velocities (right panel). In these and all subsequent kymograms and traces the tethered end of the DNA is at the top, the free end is at the bottom, and buffer flow is from top to bottom. (**C**) Histogram and (**D**) scatter plot of translocation velocities of RecBCD before and after Chi. The red line is shown as a reference with a slope of m=1.

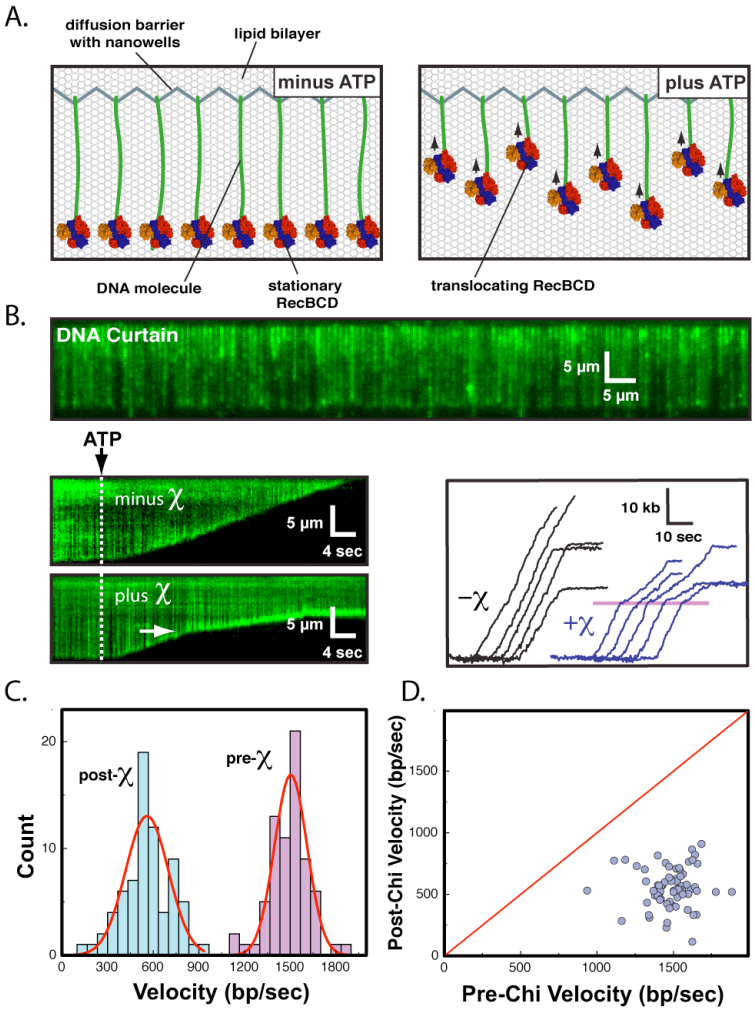

Figure S2. Single molecule and bulk characterization of RecBCD. (**A**) ATPase assays under single-molecule buffer conditions confirm that YOYO1 and the glucose oxidase oxygen scavenging system do not affect RecBCD activity (black squares: reactions without YOYO, blue triangles: with YOYO1, red circles: with YOYO1 and oxygen scavenging system, cyan: control without RecBCD, magenta: control without RecBCD but with YOYO1 and oxygen scavenging system). (**B**) Single-molecule measurement of the [ATP]-dependent RecBCD velocities at 37°C. The data is fit to a Michaelis–Menten curve with V_{max}= 1800±80 bp/sec and K_m=350±30 μM (red curve). The velocity at each ATP point is measured by fitting a histogram of at least sixty individual RecBCD traces to a Gaussian function and the error-bars correspond to the standard deviation of the fits.

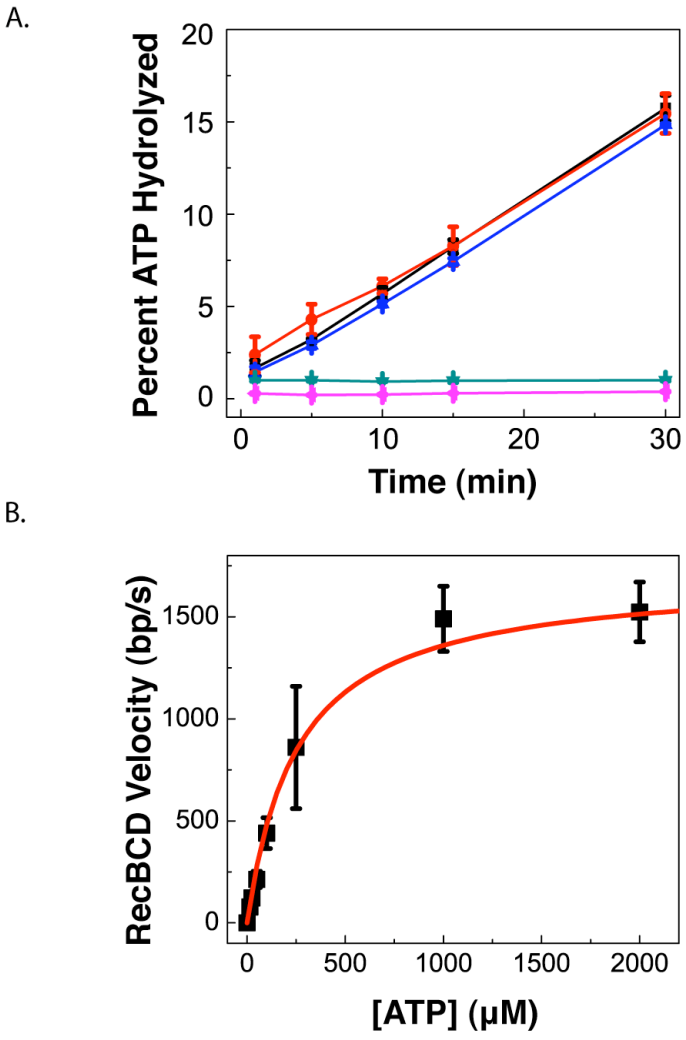

Figure S3. Single molecule and bulk characterization of RNAP. (**A**) Salt-dependent histograms of holo-RNAP bound to λ-DNA. (**B**) Histogram of core-RNAP bound to λ-DNA at 100 mM KCl. (**C**) Heparin challenge of holo- and core-RNAP on DNA curtains. Over 95% of holo-RNAP remains bound after 8 minutes. The red line is a single exponential fit with a $t_{½}$=3.4±0.03 sec (N=150). (**D**) Half-life of holo-RNAP on λ-DNA promoter regions. The red line is a single exponential fit with a $t_{½}$=23.2±1.42 min, N=58. (**E**) Transcription runoff assays confirm that epitope-tagged RNAP is fully active and that QDs do not inhibit activity. (**F**) Tracking data of individual QD-RNAPs transcribing λ-DNA in the curtain assay. Frames were acquired every 0.5 sec and smoothed with a 20 second sliding average filter. (**G**) Stalled elongation complexes were prepared on λ-DNA p_L promoter and chased with cold rNTPs to assay transcription restart efficiency. As reported previously [17], 100% of stalled complexes re-start transcription.

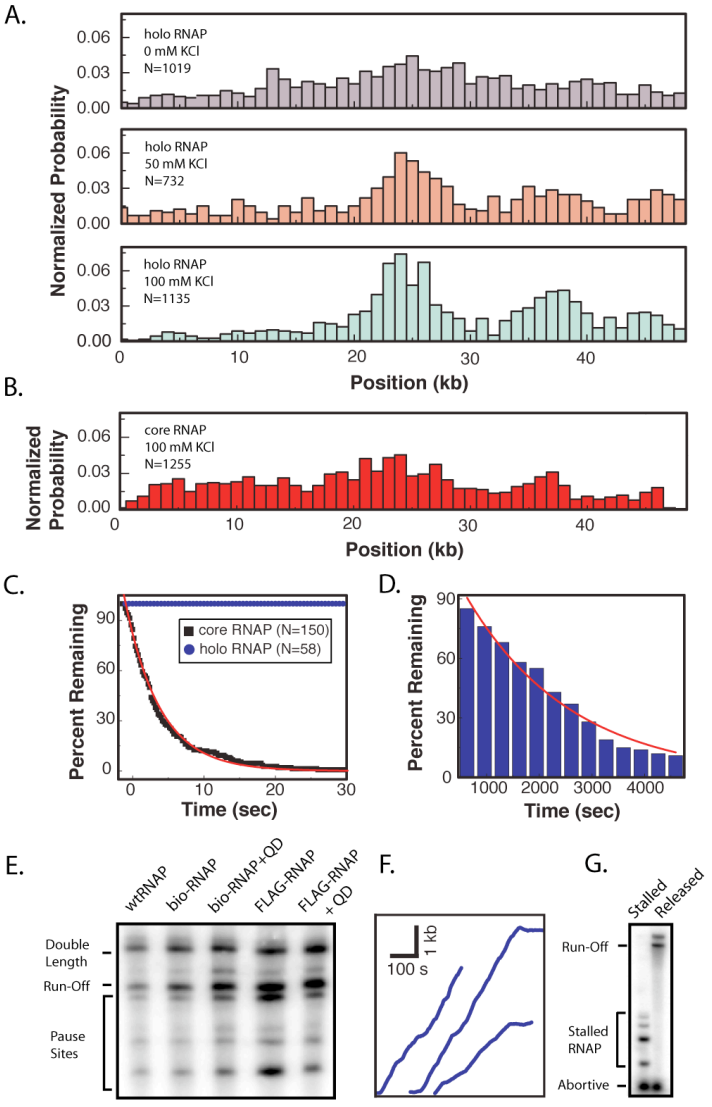

Figure S4. Analysis of Collisions with RNAP, EcoRI^{E111Q}, and nucleosomes. (**A**) Left panel, histogram of distances that RNAP holoenzyme was pushed by RecBCD. Middle panel, life times of RNAP after the collisions; blue bars represent roadblocks that were pushed along DNA, the grey bar includes life times (≤1 sec) of directly ejected roadblocks, and the red line is a single exponential fit to the data. Right panel, scatter plot showing the pre- and post-collision (while pushing RNAP) velocities of RecBCD. The red reference line has a slope of m=1. All data points fall on or near the reference line, and linear fit to the data yields a slope of m=0.994±0.02 (R^2=0.98), indicating no statistical difference in the velocity of RecBCD after the collisions (t-test, p=0.87). (**B**) Left, middle, and right panels show EcoRI^{E111Q} pushing distances, lifetime after the collisions, and a scatter plot with pre- and post-collision RecBCD velocities, respectively. All data points in the scatter plot fall on or near the reference line (m=1), and linear fit to the data (not shown) yields a slope of m=1.0±0.02 (R^2=0.99), indicating no statistical difference in the velocity as a consequence of the collisions (t-test, p=0.55). (**C**) Left, middle, and right panels show nucleosome pushing distances, lifetimes, and a scatter plot with pre- and post-collision RecBCD velocities, respectively. The points cluster below the reference line (m=1), and a linear fit to the data (dashed red line) yields a slope of m=0.90±0.04 (R^2=0.82), corresponding to a statistically significant 10% reduction in RecBCD velocity while pushing nucleosomes (t-test, p=0.0005).

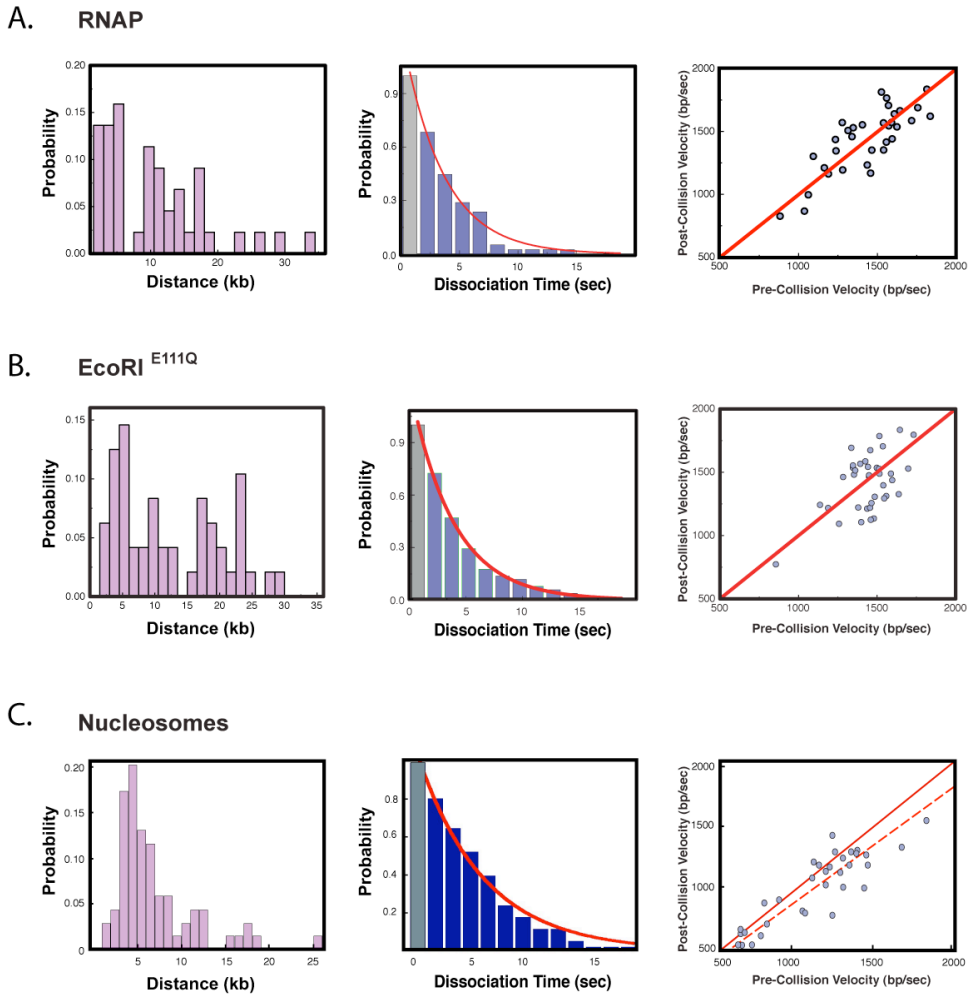

Figure S5. Analysis of collisions between RecBCD and various forms of RNAP.
Comparison of RecBCD-induced sliding and ejection behavior of various RNAP species. Stalled and elongating complexes are evicted more readily without sliding compared to holo and core RNAPs. At least fifty collisions were observed between RecBCD and each different type of RNAP complex.

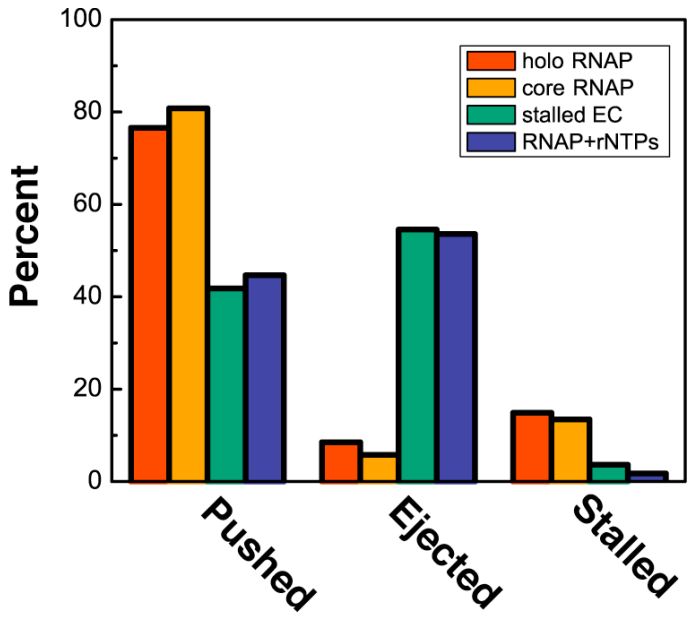

Figure S6. Collision control experiments with different fluorescent tags. Robust sliding behavior was observed for : (**A**) RNAP tagged with Alexa Fluor 488 anti-FLAG antibodies, and 40-nm polystyrene fluorescent beads (conjugated with either anti-FLAG antibodies or streptavidin); (**B**) EcoRI^{E111Q} tagged with either Alexa Fluor 488 anti-FLAG antibodies, or 40-nm polystyrene fluorescent beads conjugated with anti-FLAG antibodies; and (**C**) nucleosomes tagged with either Alexa Fluor 488 anti-FLAG antibodies, or 40-nm polystyrene fluorescent beads conjugated with anti-FLAG antibodies. Full experimental details are presented in the supplemental Materials and Methods.

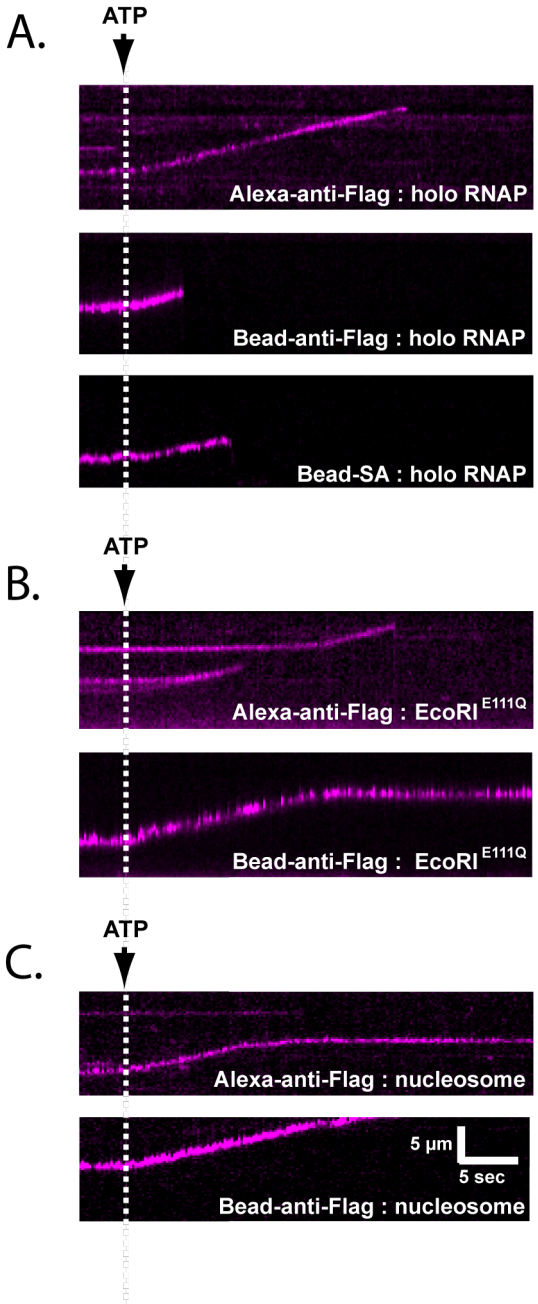

Figure S7. Reversed DNA orientation and low velocity collisions. (**A**) Kymogram of RecBCD pushing holo-RNAP on λ-DNA in a reversed orientation. (**B**) Histograms of RecBCD translocation velocities as a function of ATP concentration. (**C**) Kymograms of RecBCD colliding with QD-tagged holo-RNAP at 100 μM and 15 μM ATP concentration (as indicated). For experiments at 100 μM ATP, 0.2 sec frames were acquired every 0.4 seconds. Experiments at 15 μM ATP were conducted by acquiring 0.3 second frames every 1.3 seconds.

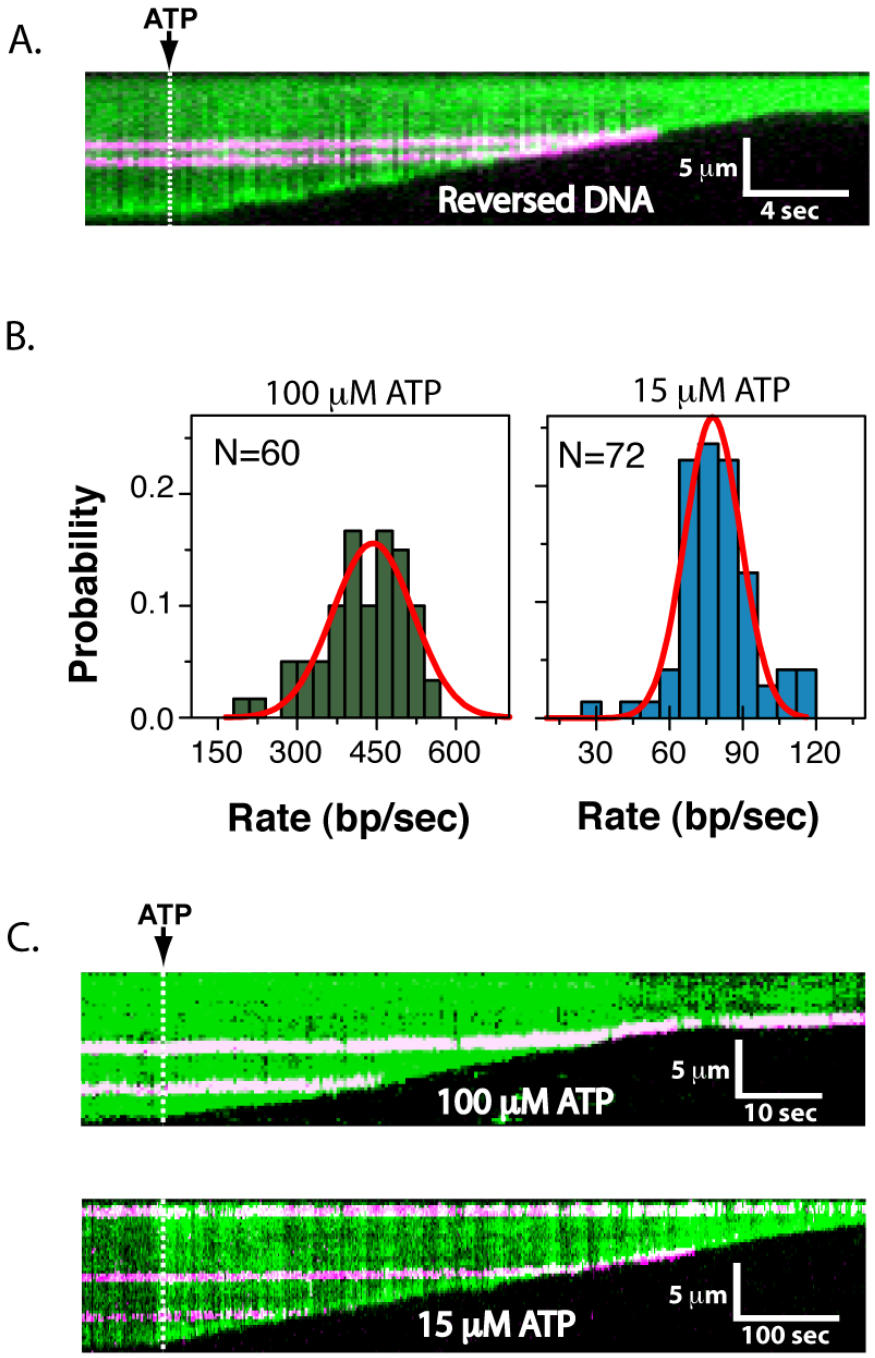

Figure S8. Bulk and single molecule characterization of EcoRI^{E111Q}. (**A**) QD-labeled EcoRI^{E111Q} bound to λ-DNA protects all five cognate sites from digestion by wild-type EcoRI. (**B**) Half-life of EcoRI^{E111Q} on λ-DNA curtain cognate sites. The red line is a single exponential fit with a $t_{½}$=18.5±1.07 minutes, N=102.

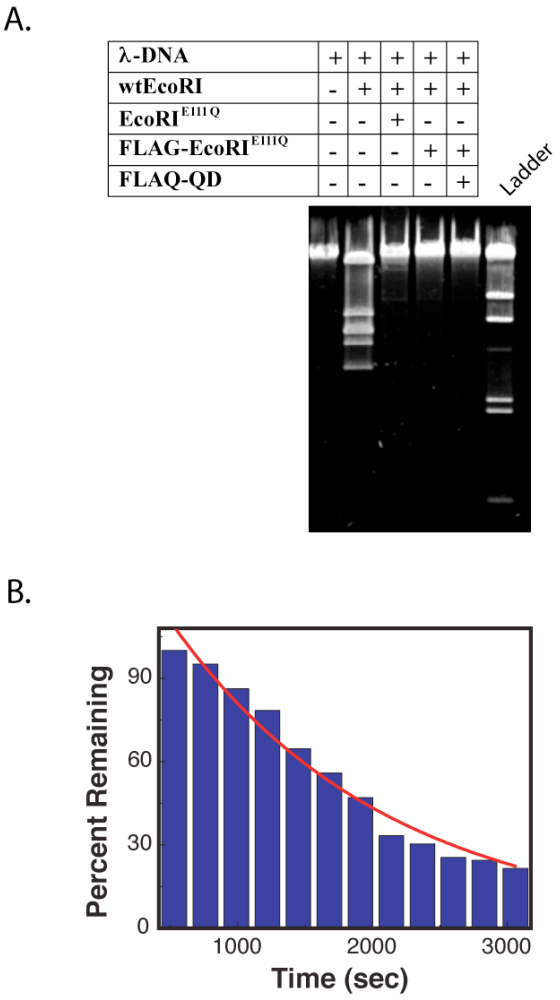

Figure S9. Characterization of *lac* repressor. (**A**) LacI bound to the LacO region of λ-DNA and 98% of molecules dissociated from the DNA in the presence of 1 mM IPTG. (**B**) Half-life of LacI on λ-DNA curtain LacO sites. The red line is a single exponential fit with a $t_{½}$=11.5±0.3 seconds, N=79. (**C**) Half-life of LacI on λ-DNA curtain LacO sites in the absence of IPTG. The red line is a single exponential fit with a $t_{½}$= 1100 ± 269 seconds, N=45.

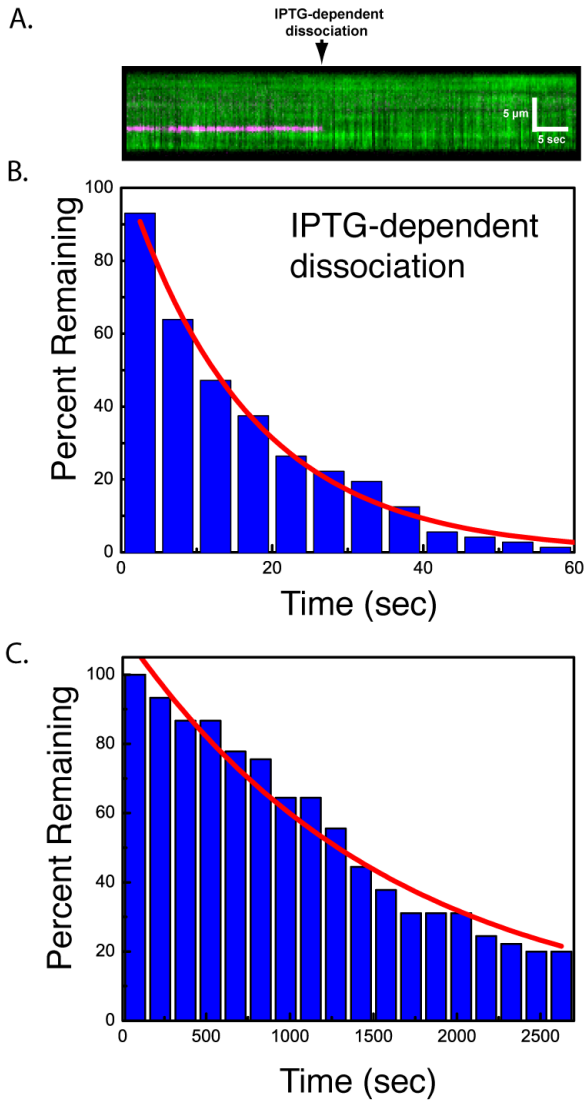

Table S1: Collision Data at Different RecBCD Velocities.

	EcoRI^{E111Q}		**holo RNAP**		**Nucleosomes**	
[ATP] (μM)	Sliding Dist. (kb)	$t_{1/2}$ (sec)	Sliding Dist. (kb)	$t_{1/2}$ (sec)	Sliding Dist. (kb)	$t_{1/2}$ (sec)
15	10.2±8.4 (N=62)	95±3 (N=45)	17.2±12.7 (N=51)	226±5.3 (N=30)	10.7±6.2 (N=63)	89±3.6 (N=24)
25	13.5±9 (N=54)	75±1.3 (N=47)	18.4±12.4 (N=39)	90±2.2 (N=38)	8.9±5.6 (N=64)	42±2.8 (N=23)
100	9.1±6 (N=55)	4±0.2 (N=40)	12±9.3 (N=65)	17±1 (N=45)	8.7±4.7 (N=65)	12.5±0.4 (N=32)
1000	13±9.1 (N=48)	2.7±0.1 (N=45)	10.5±7.7 (N=44)	2.4±0.1 (N=35)	7.3±5.4 (N=75)	3.7±0.1 (N=33)

Half-lives are reported in seconds (sec) and pushing distances are reported in kilobases (kb) at each of the four different ATP concentrations tested. See figure 5d-e for a graphical representation of the data.

Table S2: Oligonucleotides.

Name	Sequence
IF01	CGGCATCAGGCCATGGATTACAAAGATGACGACGATAAGGATTACAAAGATGACGACGATAAGGATTACAAAGATGACGACGATAAGGCTGCCGCAATGTCTAATAAAAAACAGTC
IF02	TTTATAGCTCTTCCGCACTTAGATGTAAGCTG
IF03	[P]GTACGATTACAAAGATGACGACGATAAGGATTACAAAGATGACGACGATAAGGATTACAAAGATGACGACGATAAGTAAC
IF04	[P]TCGAGTTACTTATCGTCGTCATCTTTGTAATCCTTATCGTCGTCATCTTTGTAATCCTTATCGTCGTCATCTTTGTAATC
IF05	[P]TCGAAGCTGGTGGACTAGTAGCTGCTGGTGGTTAATTAACTGCTGGTGGA
IF06	[P]CTAGTCCACCAGCAGTTAATTAACCACCAGCAGCTACTAGTCCACCAGCT
IF07	[P]AGGTCGCCGCCC[B]
IF08	[P]GGGCGGCGACCT[B]
IF09	TCAGATCTCTCACCTACCAAAC
IF10	AGGGCGGTTAACTGGTTTTG
IF11	CTCAGATTTCATGAAACCAGTAACGTTATACG
IF12	ACTCTACTAGTGTCATCTCCCGTGATGCACGCATCGATTAACTGCCCGCTTTCCAGTC
IF13	[P]TCGAATGTGTGGAATTGTGAGCGCTCACAATTCCACACAACTAGTATGAGCTTAATTAAA
IF14	[P]CTAGTTTAATTAAGCTCATACTAGTTGTGTGGAATTGTGAGCGCTCACAATTCCACACAT

† Phosphates at 5' positions are marked with a [P]. Biotin at the 3' end is marked with a [B].

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