Single-Turnover Kinetics of Helicase-Catalyzed DNA Unwinding Monitored Continuously by Fluorescence Energy Transfer†

Keith P. Bjornson, Mohan Amaratunga,‡ Keith J. M. Moore, and Timothy M. Lohman*

Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, 660 South Euclid Avenue, St. Louis, Missouri 63110

Received July 28, 1994; Revised Manuscript Received September 13, 1994⊗

ABSTRACT: We describe a fluorescence assay that can be used to monitor helicase-catalyzed unwinding of duplex DNA continuously in real time. The assay is based on the observation that fluorescence resonance energy transfer (FRET) occurs between donor (fluorescein) and acceptor (hexachlorofluorescein) fluorophores that are in close proximity due to their covalent attachment to the 3′ and 5′ ends of the complementary strands of a duplex oligodeoxynucleotide. FRET results in a reduction in the fluorescence emission intensity of fluorescein in the duplex DNA substrate relative to that observed for fluorescein-labeled single stranded DNA. Therefore, an enhancement of fluorescein fluorescence (λ_{ex} = 492 nm; λ_{em} = 520 nm) occurs upon helicase-catalyzed unwinding of the duplex DNA and separation of the complementary strands. The fluorescence assay is extremely sensitive, allowing DNA unwinding reactions to be monitored continuously at DNA concentrations as low as 1 nM in a fluorescence stopped-flow experiment. We demonstrate the use of this DNA substrate in pre-steady state, single turnover studies of duplex DNA unwinding catalyzed by the *Escherichia coli* Rep helicase, monitored by fluorescence stopped flow. We show that the fluorescence enhancement monitors Rep-catalyzed DNA unwinding by comparisons with identical kinetic studies carried out using rapid chemical quench-flow techniques. Single turnover kinetic studies performed at 1 nM DNA as a function of excess Rep concentration show that Rep-catalyzed unwinding of an 18 base pair duplex containing a 3′-ss-$(dT)_{20}$ tail is biphasic and can be described by the sum of two exponential terms. The observed rate constant of the first phase is independent of [Rep] (20−300 nM) and measures the rapid single turnover unwinding of the duplex DNA by Rep dimers bound in productive complexes (1.3 ± 0.2 s^{-1}; 23 ± 3 base pairs s^{-1} at 25.0 °C). The observed rate constant for the second phase increases linearly with [Rep], reflecting DNA unwinding that is limited by a Rep binding event occurring with a bimolecular rate constant of (1.8 ± 0.1) × 10^5 M^{-1} s^{-1}, which may reflect the rate constant for Rep dimerization on DNA. Kinetic competition studies indicate that both Rep subunits are bound stably to the DNA substrate in the productive complex that is unwound in the fast phase. The results of these kinetic studies are consistent with an active, rolling mechanism for Rep-catalyzed unwinding of DNA [Wong, I., & Lohman, T. M., (1992) *Science 256*, 350]. This fluorescence assay should greatly facilitate further mechanistic studies of helicase-catalyzed DNA unwinding.

DNA helicases are essential enzymes that unwind duplex DNA to yield the single stranded (ss) DNA intermediates required for most DNA metabolic processes, including DNA replication, recombination, and repair [for reviews see Geider and Hoffmann-Berling (1981), Matson and Kaiser-Rogers (1990), and Lohman (1992, 1993)]. During the course of unwinding DNA, helicases must also translocate along the DNA, with both processes somehow coupled to the binding and hydrolysis of nucleoside 5′-triphosphates (e.g., ATP) (Hill & Tsuchiya, 1981; Lohman, 1992, 1993). These enzymes have been isolated from both prokaryotic and eukaryotic organisms as well as many bacteriophages and viruses (Matson & Kaiser-Rogers, 1990; Thommes & Hubscher, 1992) and appear to be ubiquitous. Furthermore, it appears likely that most organisms encode multiple helicases which function selectively in various DNA metabolic processes. However, in spite of their importance, there is still relatively little known about their mechanism(s) of action, although there has been some recent progress in this area (Wong & Lohman, 1992; Amaratunga & Lohman, 1993; Lohman, 1993; Geiselmann et al., 1993).

An understanding of the mechanism(s) by which helicases catalyze duplex DNA unwinding requires knowledge of the functionally active forms of these enzymes [some are hexamers, others are dimers (Lohman, 1993)], their equilibrium binding properties to DNA (both ss and duplex) and nucleotides (ATP, ADP, etc.), and finally transient (pre-steady state) kinetic studies of ATP binding, hydrolysis, and DNA unwinding. Recently, we have used rapid chemical quench-flow techniques to examine aspects of the mechanism of DNA unwinding catalyzed by the *Escherichia coli* Rep helicase (Amaratunga & Lohman, 1993), a 3′ to 5′ helicase. These single turnover kinetic studies of Rep-catalyzed

† This work was supported in part by grants to T.M.L. from the NIH (GM 45948) and the American Cancer Society (NP-756B).

* Address correspondence to this author at the Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, 660 S. Euclid Ave., St. Louis, MO 63110. Tel: (314)-362-4393; FAX: (314)-362-7183.

‡ Present address: Parke-Davis/Warner Lambert, 2800 Plymouth Rd., Ann Arbor, MI 48105.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1994.

0006-2960/94/0433-14306$04.50/0

unwinding of radioactively labeled duplex oligodeoxynucleotides were performed over times as short as 2 ms and provide strong support for the proposal that a Rep dimer, which appears to be the functionally active form of the helicase (Chao & Lohman, 1991), unwinds DNA by an active, rolling mechanism, in which ATP binding is coupled to translocation, whereas ATP hydrolysis is coupled to DNA unwinding (Wong & Lohman, 1992; Lohman, 1992, 1993). An essential intermediate in this mechanism is a Rep−DNA complex in which both the 3′ ss-DNA and the duplex DNA at a ss-/ds-DNA junction are bound simultaneously to the Rep dimer, one to each subunit.

Although powerful, chemical quench-flow methods are time-consuming since DNA unwinding is monitored discontinuously by performing multiple reactions that are quenched after different times, followed by gel electrophoresis of each sample to separate the ss-DNA product from duplex DNA. In order to facilitate rapid kinetic and mechanistic studies of helicase-catalyzed DNA unwinding, we have designed a fluorescently labeled duplex oligodeoxynucleotide substrate that can be used to monitor helicase-catalyzed DNA unwinding continuously and in real time. The fluorescence assay is based on the observation that fluorescence resonance energy transfer (FRET) occurs between donor (fluorescein) and acceptor (hexachlorofluorescein) fluorophores covalently attached to the 3′ and 5′ ends, respectively, of a duplex oligodeoxynucleotide, whereas this energy transfer is lost upon DNA unwinding and separation of the complementary strands. Therefore, the enhancement of fluorescein fluorescence that accompanies helicase-catalyzed DNA unwinding can be used to monitor the reaction. The fluorescence assay is extremely sensitive, allowing DNA unwinding reactions to be monitored continuously at DNA concentrations as low as 1 nM. We demonstrate the use of this DNA substrate to monitor the pre-steady state unwinding of duplex DNA catalyzed by the *E. coli* Rep helicase in fluorescence stopped-flow studies and report mechanistic studies of this reaction.

MATERIALS AND METHODS

Buffers and Rep Protein. Buffers were made with reagent grade chemicals using distilled H_2O that was deionized using a Milli-Q System (Millipore Corp., Bedford, MA). Buffer U is 20 mM Tris (titrated to pH 7.5 at the indicated temperature), 6 mM NaCl, 1.7 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol. Glycerol (spectrophotometric grade) was from Aldrich (Milwaukee, WI). *E. coli* Rep protein was purified to >99% homogeneity as described (Lohman et al., 1989) and its concentration determined spectrophotometrically, using an extinction coefficient for the monomer of $\epsilon_{280} = 7.68 \times 10^4$ M^{-1} cm^{-1} (Amaratunga & Lohman, 1993).

Preparation of DNA Unwinding Substrates. Oligodeoxynucleotides were synthesized using an ABI model 391 automated DNA synthesizer (Applied Biosystems, Foster City, CA) with standard β-cyanoethyl phosphoramidite chemistry. The DNA unwinding substrates used in these studies are shown schematically in Figure 1 and consist of an 18 base pair duplex with a 3′-$(dT)_{20}$ tail. The sequence of the top strand of the duplex is 5′-GCCTCGCTGC-CGTCGCCA-3′ (Amaratunga & Lohman, 1993). The DNA substrate used in the fluorescence assay (substrate I in Figure 1) has fluorescein (F) covalently attached to the 3′ end of the top strand and hexachlorofluorescein (HF) covalently attached to the 5′ end of the bottom strand. DNA substrate II differs from I in that only the top strand is modified with fluorescein, whereas DNA substrate III has no fluorescent modification. The fluorescent oligodeoxynucleotides were prepared as follows. The top strand, containing fluorescein at its 3′ end, was synthesized using fluorescein-CPG (Glen Research, Sterling, VA) as the column support. The bottom strand contains the 18 nucleotide sequence that is complementary to the top strand with an additional $(dT)_{20}$ tail on the 3′ end (Amaratunga & Lohman, 1993) and was prepared using Expedite phosphoramidites (Millipore, Bedford, MA). Hexachlorofluorescein was added to the 5′ end of the bottom strand using the hexachlorofluorescein phosphoramidite (Applied Biosystems, Foster City, CA). The Expedite phosphoramidites were used to prepare the bottom strand since these require shorter times for deprotection and thus prevent degradation of the hexachlorofluorescein during this step. Oligodeoxynucleotides were deblocked and purified to >99% homogeneity by polyacrylamide gel electrophoresis (PAGE) as described (Amaratunga & Lohman, 1993). The concentrations of the oligodeoxynucleotides were determined by absorbance at 260 nm after correction for the contribution due to the fluorophores ($A_{260}/A_{492} = 0.3$ for fluorescein; $A_{260}/A_{535} = 0.3$ for hexachlorofluorescein). The extinction coefficient used for the top strand was 184 040 M^{-1} cm^{-1} and 365 740 M^{-1} cm^{-1} for the bottom strand. A 20 μM working stock of double stranded DNA was prepared by mixing equal concentrations of each fluorescently labeled oligonucleotide in 10 mM Tris·HCl (pH 7.5 at 25 °C), 100 mM NaCl, and 1 mM $MgCl_2$, followed by heating for 3 min at 90 °C and cooling slowly to 20 °C.

Rapid Chemical Quench-Flow Kinetics. Rapid chemical quench experiments were carried out using a three syringe pulsed quench-flow apparatus (KinTek RQF-3, University Park, PA) essentially as described (Amaratunga & Lohman, 1993) except that the Rep protein and DNA were preincubated in buffer U (rather than 2× buffer U) and the ATP was also in buffer U.[1] Rep protein and DNA [2 nM; labeled at the 5′ end of the top strand with ^{32}P as described (Amaratunga & Lohman, 1993)] were preincubated in buffer U for 10 min on ice and loaded in one loop (45 μL) of the rapid quench. The second loop (45 μL) contained 3 mM ATP in buffer U. The samples were then incubated in the loops for 4 min at 25.0 °C. The reaction was initiated by rapidly mixing the two reactants and then quenching with 0.33% SDS and 13.3% glycerol (v/v) (final concentrations) after time intervals ranging from 2 ms to 120 s. The final concentrations of DNA and ATP after mixing were 1 nM and 1.5 mM, respectively. The quenched samples were then

[1] In a previous study (Amaratunga & Lohman, 1993) the DNA unwinding reaction was also found to be biphasic, with the fast phase independent of Rep concentration. However, the rate constant for the fast phase was only 0.16 ± 0.02 s^{-1}, a factor of 10 lower than reported here. Although both studies were performed under the same final conditions, the Rep−DNA complexes in the previous study were preincubated in twice the final concentration of buffer U, containing 20% (v/v) glycerol, and the ATP stock was in water (neutralized to pH 7.5), whereas the studies reported here were performed with all components preincubated in buffer U [containing 10% (v/v) glycerol]. The different kinetics resulting from these two methods of initiating the experiment are observed in both the rapid quench and the stopped-flow experiments. We are currently investigating the effects of glycerol concentration to determine the basis for this difference.

subjected to polyacrylamide gel electrophoresis to separate the [^{32}P]-duplex DNA from the [^{32}P]-ss-DNA (20% PAGE). A DNA concentration of 1 nM was used to ensure that no renaturation of unwound DNA occurred before the samples were analyzed by gel electrophoresis (Amaratunga & Lohman, 1993) (see also Discussion). The radioactivity within each band was determined by direct imaging of the gel using a Betascope 603 blot analyzer (Betagen, Waltham, MA), and the fraction of duplex DNA unwound at each time was calculated as described (Amaratunga & Lohman, 1993). The data were plotted and fit using KaleidaGraph (Synergy Software, Reading, PA) run on a Macintosh II computer.

Fluorescence Stopped-Flow Kinetics. Stopped-flow fluorescence experiments were carried out using a KinTek model SF-2001 (KinTek, Inc., University Park, PA) in the two syringe mode, equipped with a 75W xenon lamp (USIO, Second Source, La Verne, CA) and interfaced to a Swan 486DB computer. Fluorescein (donor) was excited at 492 nm (1.5 mm slits) and fluorescence emission was monitored at wavelengths > 520 nm using a cut-on filter (Oriel Inc. cat. no. 51300, Stratford, CT). Use of a 520 nm interference filter (Oriel Inc. cat. no. 53870, Stratford, CT) showed identical kinetic time courses but with a significantly reduced signal. All kinetic traces shown in this work are from single determinations and were analyzed using software supplied by KinTek, Inc. (version 3.0).

The protocol used to prepare the Rep and DNA samples for study by stopped-flow was identical to that used in the rapid quench experiments. The Rep protein and fluorescent DNA substrate were mixed in buffer U and preincubated on ice for 10 min. The sample was then transferred to one stopped-flow syringe and equilibrated at 25.0 °C for an additional 5 min to allow for thermal equilibration (incubations for 10 min gave identical results). The other stopped-flow syringe contained ATP (3 mM) in buffer U at 25.0 °C. 500 data points were acquired in each stopped-flow experiment. In general, to facilitate quantitative analysis of each phase of the biphasic time course, a split time base was used with 250 points acquired in the first 10 s and 250 points in the remaining time.

Steady State Fluorescence Spectra. Steady state fluorescence spectra were measured using an SLM 8000 spectrofluorometer (SLM-Aminco, Urbana, IL). The samples were excited at 492 nm (2 mm slits) and fluorescence emission spectra collected (4 mm slits) in a 1 cm square quartz cuvette temperature controlled at 25.0 °C. The fluorescence emission spectra were corrected for the wavelength dependence of the photomultiplier using the SLM 8000 software. The excitation spectra were corrected for the wavelength dependence of the lamp output using a rhodamine quantum counter (15 mg/ml rhodamine B in ethanol) with front-face geometry (Jameson, 1984).

RESULTS

Duplex Formation Monitored by Fluorescence Resonance Energy Transfer between Donor and Acceptor Fluorophores on Complementary DNA Strands. A schematic representation of the fluorescently labeled DNA used to monitor helicase-catalyzed DNA unwinding is shown in Figure 1 (substrate I). This DNA substrate is based on those used in our previous chemical quench-flow studies of Rep-catalyzed unwinding, which showed that Rep helicase requires only a

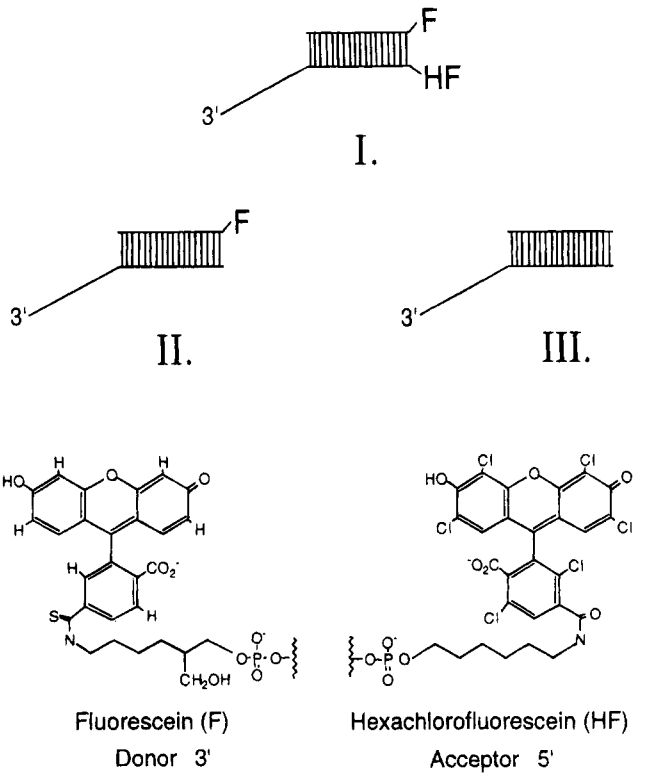

FIGURE 1: Schematic representation of the fluorescent DNA unwinding substrate I. The duplex region is 18 base pairs long (top strand sequence: 5'-GCCTCGCTGCCGTCGCCA-3'). The top strand as depicted has the fluorescein (F) donor covalently attached to the 3' end. The bottom strand has a $(dT)_{20}$ (oligodeoxythymidine) tail covalently attached to the 3' end and a hexachlorofluorescein (HF) acceptor covalently attached to the 5' end. DNA substrate II differs from DNA substrate I in that the bottom strand is not labeled with HF. DNA substrate III differs from I in that neither strand has been fluorescently modified.

3'-ss-DNA tail in order to initiate unwinding *in vitro* (Amaratunga & Lohman, 1993). The DNA substrate contains a $(dT)_{20}$ single stranded region attached to the 3' end of an 18 base pair duplex (top strand sequence of 5'-GCCTCGCTGCCGTCGCCA-3'); this is the same duplex sequence used in our previous studies (Amaratunga & Lohman, 1993). The top strand of DNA (I) has fluorescein (F) (the fluorescence donor in the FRET pair) covalently attached to its 3' end via a six carbon linker, whereas the bottom strand has hexachlorofluorescein (HF) (the fluorescence acceptor) covalently attached to its 5' end also via a six carbon linker. Figure 1 also depicts DNA substrates II and III, which differ from substrate I only in the fluorescent modification and were used as controls for the present study. In DNA substrate II, only the top strand is modified with fluorescein, whereas DNA substrate III contains no fluorescent modifications.

Figure 2 compares the fluorescence excitation and emission spectra of the individual single strands labeled with either fluorescein (F) (top strand) or hexachlorofluorescein (HF) (bottom strand). This comparison shows the spectral characteristics and spectral overlap that make the fluorescein and hexachlorofluorescein pair useful for FRET studies. The maximum excitation wavelength for F is 497 nm, whereas its maximum emission wavelength is 524 nm. The maximum excitation wavelength for HF is 538 nm, whereas its maximum emission wavelength is 551 nm. Most significantly, the emission spectrum of F shows considerable

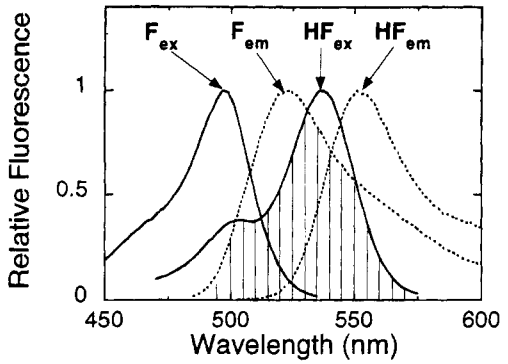

FIGURE 2: Fluorescence spectral characteristics of fluorescein (donor) and hexachlorofluorescein (acceptor). Fluorescence excitation (continuous lines) and emission (dashed lines) spectra were determined (buffer U, 25.0 °C) for the individual top single stranded oligodeoxynucleotide 3′-labeled with fluorescein (F) (10 nM) and the bottom single stranded oligodeoxynucleotide 5′-labeled with hexachlorofluorescein (HF) (10 nM) (values of λ_{max} for F_{ex}, F_{em}, HF_{ex}, and HF_{em} are 497, 524, 538, and 551 nm, respectively). The shaded area represents the spectral overlap between the fluorescently labeled DNA donor emission (F_{em}) and the acceptor excitation (HF_{ex}), which is required for FRET.

overlap with the excitation spectrum of HF (shaded area). Therefore, if the F and HF fluorophores are in close proximity, upon excitation of F, nonradiative transfer of energy from F to HF can occur, which will then be emitted by HF, resulting in a decrease in fluorescence emission from F.

To determine whether fluorescence energy transfer from fluorescein (donor) to hexachlorofluorescein (acceptor) occurs upon formation of duplex DNA, we compared the fluorescence emission spectra of the individual top and bottom single strands to a 1:1 mixture of both strands to form the duplex DNA substrate I (see Figure 1). These emission spectra are shown in Figure 3A for DNA strand concentrations of 10 nM using an excitation wavelength of 492 nm (to excite fluorescein). Spectrum 1 is the emission spectrum of the top strand with fluorescein (donor) attached to its 3′ end, whereas spectrum 2 is the emission spectrum of the same top strand hybridized to the complementary bottom strand which has hexachlorofluorescein (acceptor) attached to its 5′ end. Comparison of these two spectra shows a ~3-fold decrease in fluorescein emission intensity at 520 nm due to resonance energy transfer from fluorescein to hexachlorofluorescein upon formation of duplex DNA. Spectrum 3 in Figure 3A is the emission spectrum of the bottom strand containing the HF acceptor (excited at 492 nm), in the absence of the top strand, to show its minimal contribution to the fluorescence emission intensity at 520 nm upon excitation at 492 nm. These spectra indicate that a measurable increase in fluorescein emission intensity should accompany the unwinding of the duplex DNA substrate I shown in Figure 1.

Figure 3B shows the change in fluorescence emission spectrum after duplex DNA I is unwound by the Rep helicase. In these experiments, Rep protein (500 nM monomer) was preincubated with the fluorescent DNA I (10 nM) in buffer U at 25.0 °C, and emission spectra (λ_{ex} = 492 nm) were taken before and 4 min after the addition of ATP (1.5 mM). The spectrum was taken 4 min after ATP addition since Rep-catalyzed DNA unwinding of this substrate is complete after this time (Amaratunga & Lohman, 1993; this work), whereas only half of the 1.5 mM ATP has

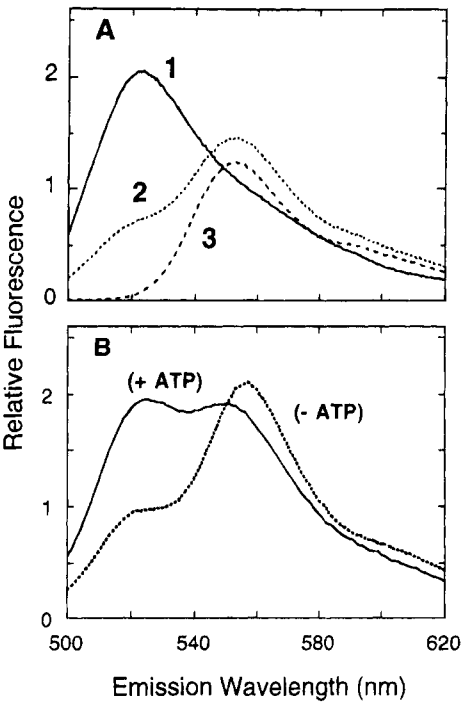

FIGURE 3: Steady state fluorescence emission spectra of the fluorescently labeled unwinding substrate and the separate single stranded oligodeoxynucleotides. (A) Fluorescence emission spectra taken in the absence of Rep protein in buffer U at 25 °C (λ_{ex} = 492 nm). (1) Single stranded oligodeoxynucleotide 3′-labeled with fluorescein (F) (10 nM) (top strand); (2) duplex DNA substrate I (see Figure 1) with top strand 3′-labeled with fluorescein (F) and bottom strand 5′-labeled with hexachlorofluorescein (HF) (10 nM); (3) Single stranded oligodeoxynucleotide 5′-labeled with hexachlorofluorescein (HF) (10 nM) (bottom strand). (B) Fluorescence emission spectra of a solution of DNA substrate I (10 nM) and 500 nM Rep monomer in buffer U at 25.0 °C. (−ATP) Spectrum taken in the absence of ATP; (+ATP) spectrum taken 4 min after addition of 1.5 mM ATP.

been hydrolyzed (K. J. M. Moore, unpublished results). After Rep-catalyzed DNA unwinding, the relative fluorescein (donor) fluorescence increases ~2-fold at 520 nm, while there is a concomitant loss of sensitized acceptor emission fluorescence at 560 nm. After complete hydrolysis of the ATP an additional ~5% enhancement of the donor fluorescence is observed at 520 nm, whereas no additional change in acceptor fluorescence is observed at 560 nm (data not shown). These results are consistent with FRET occurring between the fluorescein donor and hexachlorofluorescein acceptor when the fluorescently labeled oligonucleotides are in the duplex form. Upon formation of the single strands there is a loss of FRET resulting in a relative fluorescence enhancement at 520 nm. We note that the ~2-fold change in fluorescence emission at 520 nm observed in the Rep-catalyzed DNA unwinding reaction (Figure 3B) is significantly lower than the ~3-fold change observed in the absence of Rep (Figure 3A).

Single-Turnover Kinetics of Rep-Catalyzed DNA Unwinding Monitored by Stopped-Flow Fluorescence. We used stopped-flow fluorescence techniques to determine whether the kinetics of Rep-catalyzed DNA unwinding could be monitored by the increase in fluorescence emission of the fluorescently labeled DNA substrate. In these experiments, the DNA substrate was pre-equilibrated with Rep protein in buffer U in one drive syringe and the reaction was initiated by rapid mixing with ATP in the same buffer. An excitation

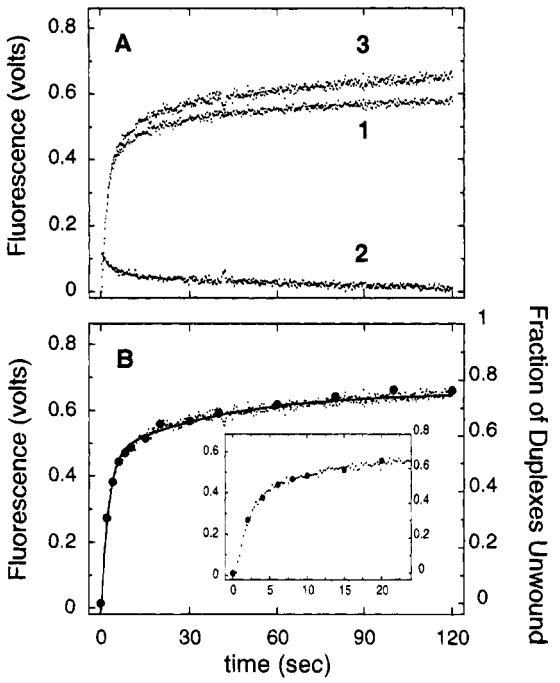

FIGURE 4: Comparison of Rep-catalyzed DNA unwinding kinetics monitored by stopped-flow fluorescence and rapid chemical quench-flow. (A) Unwinding reactions were monitored in a stopped-flow by the increase in fluorescein (donor) fluorescence (λ_{ex} = 492 nm, λ_{em} > 520 nm) as described in Materials and Methods (15.0 °C). Rep and the 18 base pair duplex substrate in Figure 1 were preincubated in buffer U, and the reaction was initiated by rapid mixing with ATP. Final concentrations after mixing were 500 nM Rep monomer, 1 nM DNA substrate, and 1.5 mM ATP. All stopped-flow traces shown are from single determinations. (1) Stopped-flow fluorescence time course for DNA substrate I (see Figure 1) with top strand labeled at the 3′ end with fluorescein (F) donor and the bottom strand labeled at the 5′ end with hexachlorofluorescein (HF) acceptor; (2) stopped-flow fluorescence time course for DNA substrate II (see Figure 1) with only top strand labeled at the 3′ end with fluorescein (F) donor; (3) Time course 2 subtracted from time course 1. (B) Stopped-flow fluorescence kinetics (curve 3 from panel A) (left axis) compared with the DNA unwinding kinetics determined using chemical quench-flow (●) (right axis) for the same DNA substrate under identical conditions. In the quench-flow experiment, the top strand was labeled on its 5′ end with ^{32}P. The smooth curve is a simulated time course, based on eq 1 and the parameters $k_{obs,1}$ = 0.43 ± 0.01 s^{-1}, A_1 = 0.525 ± 0.007 V, $k_{obs,2}$ = 0.026 ± 0.001 s^{-1}, and A_2 = 0.194 ± 0.003 V, which were determined from nonlinear least squares regression analysis of the stopped-flow data (buffer U, 15.0 °C). The inset compares the stopped-flow fluorescence and chemical quench-flow kinetics over the first 25 s.

wavelength of 492 nm was used, and fluorescence emission was monitored at wavelengths >520 nm using a 520 nm cut-on filter. The results of stopped-flow experiments performed at 15 °C at final concentrations of 500 nM Rep (monomer), 1 nM DNA duplex, and 1.5 mM ATP are shown in Figure 4. Curve 1 shows a significant fluorescence enhancement with a biphasic time course after addition of ATP to the pre-equilibrated solution of Rep and DNA substrate I (Figure 1). Curve 2 shows the time course of the fluorescence change for a control experiment using DNA substrate II (Figure 1) in which only the top strand of the duplex was labeled with the fluorescein donor. This control indicates that in the absence of HF no enhancement of the fluorescein acceptor occurs upon addition of ATP and subsequent DNA unwinding. On the contrary, curve 2 shows a small biphasic decrease in fluorescence, which cannot be due to FRET, but which must also contribute to the fluorescence change observed in curve 1. Therefore, we have subtracted curve 2 from curve 1 to obtain the final time course of DNA unwinding shown in curve 3.[2]

To determine whether the fluorescence stopped-flow trace (curve 3 in Figure 4) is an accurate reflection of the time course of DNA unwinding, we examined the kinetics of Rep-catalyzed DNA unwinding of the fluorescently labeled DNA substrate I under identical solution conditions and protein and DNA concentrations, but using chemical quench-flow methods. For these experiments, the top strand of the duplex was labeled on its 5′ end with ^{32}P, and the products of unwinding were separated by polyacrylamide gel electrophoresis. The time course of Rep-catalyzed DNA unwinding determined by chemical quench-flow is shown in Figure 4B (filled circles), along with the fluorescence stopped-flow time course (curve 3 from Figure 4A). In Figure 4B, the total fluorescence change observed in the stopped-flow experiment was normalized to the observed fraction of duplexes unwound in the quench-flow experiment. The time courses determined by these two methods are superimposable, demonstrating that the increase in fluorescein fluorescence associated with the loss of FRET can be used continuously and in real time to monitor the kinetics of Rep-catalyzed unwinding of the DNA substrate in Figure 1.

The kinetics of DNA unwinding are biphasic as shown in Figure 4B, and the time course can be described as a sum of two exponential terms as in:

$$A(t) = A_1[1 - \exp(-k_{obs,1}t)] + A_2[1 - \exp(-k_{obs,2}t)] \quad (1)$$

where $A(t)$ is the total amplitude at time t. The first phase of unwinding, referred to as the burst, occurs with observed rate constant $k_{obs,1}$ and amplitude A_1 and is well resolved from a slower phase occurring with rate constant $k_{obs,2}$ and amplitude A_2. The amplitudes of the stopped-flow fluorescence and quench-flow data are given in volts (V) and fraction of duplexes unwound, respectively. The solid line in Figure 4B shows the nonlinear least squares fit of the stopped-flow fluorescence time course to eq 1, with $k_{obs,1}$ = 0.43 ± 0.01 s^{-1}, A_1 = 0.525 ± 0.007 V, $k_{obs,2}$ = 0.026 ± 0.001 s^{-1}, and A_2 = 0.194 ± 0.003 V (15 °C, buffer U). Equation 1 provides a better description of the stopped-flow data than does an exponential plus a steady state phase, which was used previously by our laboratory to describe the time courses of Rep-catalyzed DNA unwinding determined solely by rapid quench-flow methods (Amaratunga & Lohman, 1993). The many more data points that can be obtained in the second phase using the stopped-flow experiment (compared to the quench-flow experiment) enable us to better differentiate between the suitability of these two descriptions of the time course.

[2] Stopped-flow fluorescence experiments performed with DNA substrate II containing a 20% excess of the bottom strand over the fluorescein-labeled top strand showed the identical fluorescence time course. A further control experiment showed that no fluorescence change (λ_{ex} = 492 nm, λ_{em} ≥ 520 nm) was associated with Rep-catalyzed unwinding of a DNA substrate labeled with only the acceptor fluorophore, HF. Thus, signal changes due to direct excitation of HF do not contribute to the observed fluorescence signal in the DNA unwinding reaction.

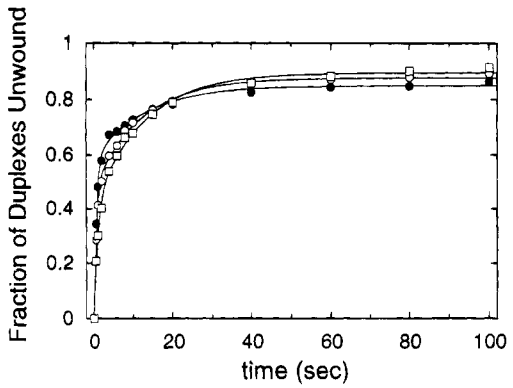

FIGURE 5: Effect of FRET fluorophores on the kinetics of Rep-catalyzed unwinding of 18 base pair DNA substrate measured by rapid quench-flow. Experiments were performed at final concentrations of 300 nM Rep and 1 nM duplex DNA substrate (top strand ^{32}P-labeled), 1.5 mM ATP in buffer U at 25.0 °C. (●) DNA substrate I (see Figure 1) with top strand modified with fluorescein (F) and bottom strand modified with hexachlorofluorescein (HF); (○) DNA substrate II (see Figure 1), with only top strand modified with fluorescein (F) (donor only); (□) unmodified DNA substrate III (see Figure 1). The nonlinear least squares fits of the data to eq 1 are shown, and the kinetic parameters are given in Table 1.

Table 1: Effects of Fluorescent Modification of DNA Substrate on Rep-Catalyzed DNA Unwinding Kinetics Measured by Rapid Quench[a]

DNA substrate from Figure 1	A_1 (fraction of duplexes unwound)	$k_{obs,1}$ (s^{-1})	A_2 (fraction of duplexes unwound)	$k_{obs,2}$ (s^{-1})
I	0.58 ± 0.02	1.6 ± 0.2	0.27 ± 0.02	0.080 ± 0.010
II	0.48 ± 0.02	1.6 ± 0.2	0.40 ± 0.02	0.080 ± 0.007
III	0.41 ± 0.03	1.0 ± 0.2	0.49 ± 0.03	0.080 ± 0.009

[a] Experiments were performed in buffer U, 25.0 °C, by rapid chemical quench-flow as described in Materials and Methods, and the data were fit to eq 1 to obtain the kinetic parameters.

To determine whether the fluorophores have any effect on the kinetics of Rep-catalyzed DNA unwinding of the 18 base pair DNA substrate, we compared the unwinding kinetics of the three DNA substrates I, II, and III shown in Figure 1. These experiments were performed at 25.0 °C in buffer U using rapid chemical quench-flow methods by preincubating Rep and DNA (300 nM Rep, 1 nM DNA, final concentrations) and initiating the reaction by addition of ATP (1.5 mM final concentration). Figure 5 shows that all three substrates are unwound to the same final extent and with very similar biphasic kinetics. The time courses in Figure 5 were fit to eq 1, yielding the kinetic parameters given in Table 1 [note that the rate of unwinding at 25.0 °C is significantly faster than at 15.0 °C (see below)].

As shown in Table 1, the kinetics of unwinding DNA substrate I, modified with both the fluorescent donor (F) and acceptor (HF) (filled circles), and DNA substrate II, modified with only the fluorescent donor (F) (open circles), are identical within the reproducibility of the quench-flow experiment. The nonfluorescent DNA substrate III appears to be unwound with a slightly slower burst rate constant than the modified substrates, although this difference is also small. All three substrates show similar burst amplitudes and identical rate constants of unwinding in the slow phase, $k_{obs,2}$. In all three reactions the total fraction of duplexes unwound was ~0.88. Accounting for the experimental error associated with the chemical quench-flow experiments, we conclude that the three kinetic time courses shown in Figure 5 differ very slightly, if at all. The fact that DNA substrates I and II are unwound with essentially identical kinetics indicates that the correction applied to the stopped-flow fluorescence time course in Figure 4A (i.e., subtraction of curve 2 from curve 1) is valid.

Effect of Rep Protein Concentration on DNA Unwinding Kinetics. Stopped-flow and rapid quench experiments were compared at several Rep concentrations under identical solution conditions in buffer U at 25.0 °C [1 nM DNA substrate I (5′ end labeled with ^{32}P on the top strand for the rapid quench experiments), 1.5 mM ATP in buffer U]. The Rep and DNA samples in buffer U also included 100 μg/mL bovine serum albumin (BSA), which improved reproducibility in the rapid quench experiments at low Rep concentrations, presumably by reducing surface binding of Rep. Therefore, BSA was also used in the stopped-flow experiments shown in Figure 6 for comparative purposes, although we show below that the stopped-flow results are not influenced by the presence of BSA (see Figure 7A,B). Each stopped-flow unwinding reaction shown was corrected by subtracting the kinetics of the fluorescence decrease of a control reaction performed at each protein concentration with DNA substrate II as described for the experiments in Figure 4A. The reactions were initiated by the rapid mixing of ATP in buffer U (to 1.5 mM). The kinetics of the unwinding reactions determined at four Rep concentrations (50, 100, 200, and 300 nM) are compared in Figure 6. At each Rep concentration, the kinetic time course determined by stopped-flow fluorescence agrees very well with that determined by quench-flow.

The stopped-flow data were analyzed by fitting each time course to eq 1, and the observed rate constants for the two kinetic phases are plotted as a function of the Rep protein concentration (closed circles) in Figure 7A,B. Also shown in Figure 7A,B are the observed rate constants determined for a separate set of stopped-flow unwinding experiments performed in the absence of BSA (open circles), indicating the lack of influence of BSA in the stopped-flow experiments. Figure 7A shows that $k_{obs,1} = 1.3 \pm 0.2\ s^{-1}$ at 25 °C, independent of Rep concentration. Therefore, the first phase of the time course represents single turnover DNA unwinding by Rep bound to DNA in productive complexes, and $k_{obs,1}$ is the rate constant for Rep-catalyzed unwinding of the 18 base pair duplex, corresponding to an unwinding rate of 23 ± 3 base pairs s^{-1}. We also note that the value of $k_{obs,1}$ determined at 25.0 °C (1.3 ± 0.2 s^{-1}) is significantly greater than the value determined under the same conditions at 15 °C (0.43 ± 0.01 s^{-1}). In contrast to the first phase, the observed rate constant for the slow phase, $k_{obs,2}$, increases linearly with Rep concentration (0−300 nM; Figure 7B), indicating that unwinding in this slower phase is limited by the binding of additional Rep protein. The apparent bimolecular association rate constant, determined from the slope of the linear least squares line in Figure 7B, is (1.8 ± 0.1) × 10^5 M^{-1} s^{-1}. Figure 7C shows that the amplitude of the fast phase (open squares) increases with Rep concentration, whereas the amplitude of the slow phase (closed squares) decreases. The total amplitude of the fluorescence change (open diamonds) is independent of Rep concentration. The increase in amplitude of the first phase with Rep concentration, which we also observed previously (Amaratunga & Lohman, 1993), reflects the increased amount of DNA bound in productive Rep−DNA complexes, which is subsequently

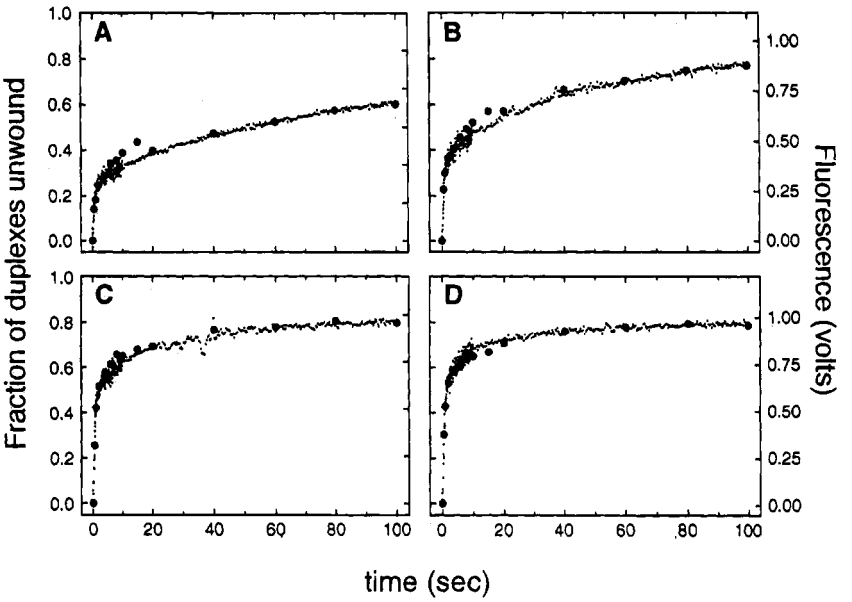

FIGURE 6: Rep protein concentration dependence of the DNA unwinding reaction monitored by fluorescence stopped-flow and chemical quench-flow. Unwinding reactions were performed using rapid quench (●) (fraction of duplexes unwound; left axis) and stopped-flow fluorescence (volts; right axis) under identical conditions and concentrations. The stopped-flow traces shown have been corrected as described in Figure 4A. Rep and DNA were preincubated, and the reaction was started by rapid mixing with ATP in buffer U at 25.0 °C. The final concentrations were 1 nM fluorescently labeled DNA substrate I (top strand ^{32}P-labeled for rapid quench), 1.5 mM ATP, and (A) 50 nM Rep monomer; (B) 100 nM Rep monomer; (C) 200 nM Rep monomer; (D) 300 nM Rep monomer.

unwound upon adding ATP, and is consistent with the equilibrium constants for Rep−DNA binding and Rep dimerization (Wong et al., 1992; Wong & Lohman, 1992).

We emphasize that the single turnover rate constant for DNA unwinding, $k_{obs,1}$, is a macroscopic rate constant that may reflect multiple kinetic steps, including possible protein conformational changes, that might occur prior to the complete DNA unwinding event. In this regard, we tested the possibility that the observed rate constant, $k_{obs,1}$, reflects a slow protein conformational change occurring prior to DNA unwinding by determining whether $k_{obs,1}$ is sensitive to the length of the duplex DNA. From rapid quench-flow experiments performed with a DNA substrate containing a 40 base pair duplex and a 3′-$(dT)_{20}$ tail, we determined $k_{obs,1} = 0.56 \pm 0.04$ s^{-1} in buffer U at 25.0 °C (data not shown), which is significantly lower than the value of $k_{obs,1} = 1.3 \pm 0.2$ s^{-1} measured for the 18 base pair duplex. Therefore, $k_{obs,1}$ does reflect kinetic steps associated with DNA unwinding, and furthermore, the apparent duplex DNA unwinding rate per base pair is the same for both substrates (23 ± 3 bp s^{-1}).

The amplitude of the stopped-flow fluorescence signal (in volts) can be calibrated in terms of the extent of duplex unwinding by comparison with the quench-flow results shown in Figure 6 after fitting to eq 1. Figure 8 shows that the amplitudes from the fluorescence assay are directly proportional to the fraction of duplexes unwound determined in the quench-flow experiment. Since, under these conditions, the total amplitudes from either assay are constant (0.88 of the duplexes are unwound), independent of Rep concentration (Figure 7C and data not shown), the total amplitude of the fluorescence signal change must correspond to 0.88 fraction of duplexes unwound, independent of the particular voltage setting of the photomultiplier. Therefore, calibration of the fluorescence unwinding assay by rapid quench, which allows quantitative interpretation of the fluorescence amplitudes, needs to be performed only once for each set of solution conditions.

Effects of Competitor DNA on the Kinetics of DNA Unwinding by Preformed Rep−DNA Complexes. We have examined the effects of competitor DNA on the kinetics of the Rep-catalyzed DNA unwinding reaction to probe the two phases and to further test our conclusion that the first phase of the unwinding reaction represents the single turnover unwinding of DNA by Rep that is bound in a productive complex to DNA before addition of ATP. As before, stopped-flow unwinding experiments were performed by preincubating Rep protein and fluorescently labeled DNA substrate I (final concentrations: 50 nM Rep and 1 nM DNA), and the reaction was initiated by the rapid addition of ATP to 1.5 mM; however, in these reactions, varying amounts of competitor DNA were included in the ATP solution. This concentration of Rep was chosen since, under these conditions, the amplitudes of both phases of the unwinding time course are approximately equal (see Figure 6A). Two types of competitor DNA were used; one was single stranded $(dT)_{16}$, and the other was the same DNA substrate, but without the FRET fluorophores (III in Figure 1). Each stopped-flow trace was corrected as described above to obtain the final unwinding time course, which was then fit to eq 1 to obtain $k_{obs,1}$ and $k_{obs,2}$, which are plotted in Figure 9. Figure 9A shows that $k_{obs,1}$ is independent of the concentration of either competitor DNA. The amplitude of the fast phase was also independent of the competitor DNA concentration (data not shown). This demonstrates that $k_{obs,1}$ measures the single turnover rate of DNA unwinding by Rep bound to DNA in productive, kinetically stable complexes and that these productive complexes are not accessible by either competitor DNA. In contrast, Figure 9B shows that $k_{obs,2}$ decreases hyperbolically with increasing competitor concentration. Identical results were observed using $(dT)_{16}$ or the nonfluorescent DNA substrate as competitor. This is consistent with our conclusion that the second phase of this reaction involves the additional binding of Rep protein; hence the second phase can be eliminated by the competitor DNA

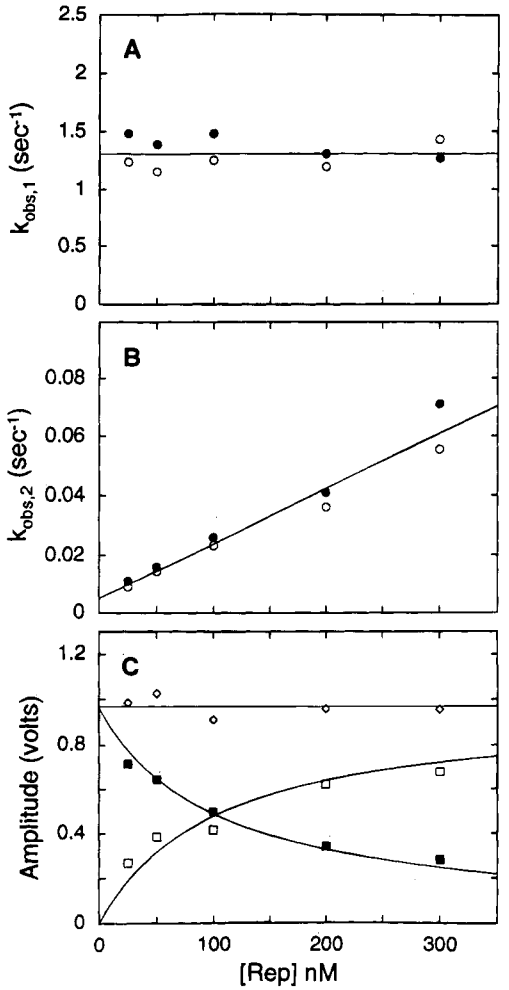

FIGURE 7: Dependence on Rep concentration of kinetic parameters for Rep-catalyzed unwinding of DNA substrate I. Kinetic parameters were obtained for the stopped-flow time courses shown in Figure 6 by nonlinear least squares fitting of the data to eq 1. (A) Observed rate constant for the fast phase ($k_{obs,1}$). (●) Experiments performed in the presence of BSA (50 μg/mL); (○) experiments performed in the absence of BSA. Horizontal line drawn at the average value of $k_{obs,1}$ = 1.3 ± 0.2 s^{-1}. (B) Observed rate constant for the slow phase ($k_{obs,2}$). (●) Experiments performed in the presence of BSA (50 μg/mL); (○) experiments performed in the absence of BSA. Linear least squares line is shown [slope = (1.8 ± 0.1) × 10^5 M^{-1} s^{-1}; intercept = 0.005 ± 0.003 s^{-1}]. (C) Amplitudes of DNA unwinding for the fast phase (□), slow phase (■), and total (◇).

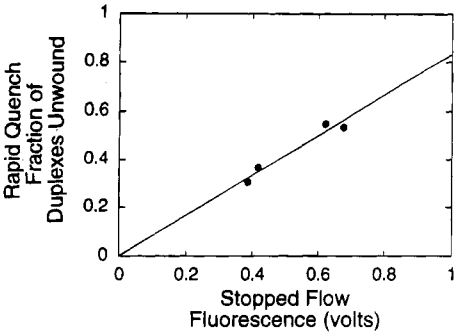

FIGURE 8: Correlation of the amplitudes of the fast phase of the Rep-catalyzed DNA unwinding reaction determined by rapid quench vs stopped-flow. Amplitude of the fast phase determined by rapid quench (fraction of duplexes unwound) is plotted vs the amplitude of the fast phase determined by fluorescence stopped-flow (volts) for the data shown in Figure 5 (slope of line is 0.83 ± 0.03 fraction duplexes unwound/V).

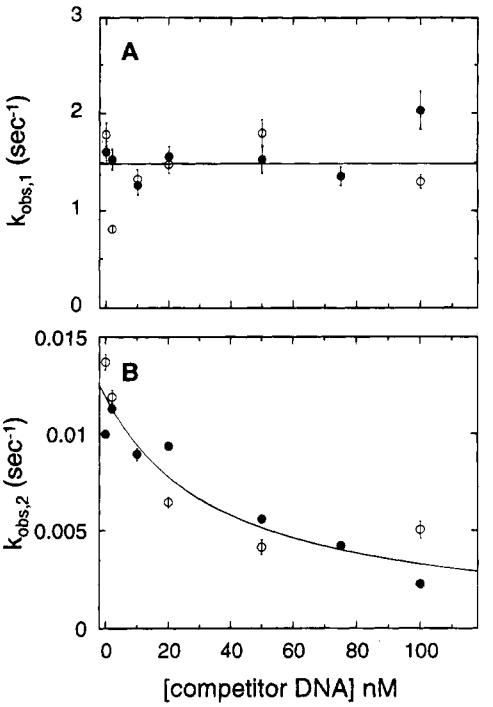

FIGURE 9: Effect of competitor DNA on the observed rate constants for Rep-catalyzed DNA unwinding. Stopped-flow fluorescence unwinding experiments were performed as described in Materials and Methods in buffer U at 25 °C [final concentrations of 50 nM Rep monomer and 1 nM DNA substrate I (Figure 1)], except that varying amounts of competitor DNA were added with the ATP (final concentration 1.5 mM). Competitor DNA was (●) $(dT)_{16}$ and (○) nonfluorescent 18 base pair duplex DNA (substrate III in Figure 1). (A) Dependence of the observed rate constant for the fast phase ($k_{obs,1}$) on competitor DNA concentration. Line drawn at the average value of 1.5 ± 0.3 s^{-1}. (B) Dependence of the observed rate constant for the slow phase ($k_{obs,2}$) on competitor DNA concentration.

that can trap free Rep protein and prevent it from binding to the fluorescent DNA substrate.

DISCUSSION

A Fluorescent Assay for Monitoring Helicase-Catalyzed DNA Unwinding. Since the pioneering studies of Stryer and Haugland (1967), Forster energy transfer between two fluorescent groups has been used to probe distances in a variety of biological macromolecules (Fairclough & Cantor, 1978; Clegg, 1992). This approach has recently been used to obtain information about distances between the ends of duplex oligodeoxynucleotides (Cooper & Hagerman, 1990; Clegg et al., 1993; Clegg, 1992), and also as a means of monitoring the reassociation kinetics of oligodeoxynucleotides (Cardullo et al., 1988). We show here that FRET between donor (fluorescein) and acceptor (hexachlorofluorescein) fluorophores covalently attached to the 3′ and 5′-ends of the two complementary strands of a duplex oligodeoxynucleotide, 18 base pairs in length, can be used to monitor continuously the kinetics of a helicase-catalyzed DNA unwinding reaction in real time by stopped-flow techniques. Due to the proximity of the fluorophores at the same blunt end of the duplex, there is efficient energy transfer from the fluorescein donor to the hexachlorofluorescein acceptor; however, energy transfer is lost upon unwinding of the duplex by the helicase, allowing the unwinding reaction to be monitored by the increase in fluorescein fluorescence. Excellent agreement between the results of the stopped-flow FRET assay and rapid quench-flow method was obtained at

both 15 and 25 °C and at several protein concentrations, indicating that the FRET assay accurately monitors unwinding of these short duplexes. Fluorescein and hexachlorofluorescein were chosen for use in our FRET experiments due to their excellent spectral characteristics; the emission spectrum of the fluorescein donor overlaps significantly the excitation spectrum of the hexachlorofluorescein acceptor. In addition, the wavelength needed to excite fluorescein (492 nm) is well removed from the absorbance bands of DNA, protein, and nucleotides, eliminating the need for inner filter corrections.

A fluorescent based assay has several obvious advantages for kinetic studies in general and particularly for mechanistic studies of helicase-catalyzed DNA unwinding that require information in the millisecond to second time domain. First of all, such an assay allows DNA unwinding to be monitored continuously in real time. In discontinuous assays, such as chemical quench-flow, each time point is taken from an individual experiment; hence multiple experiments, designed to be identical, must be repeated to obtain a complete time course. This increases the experimental variation and generally limits the number of data points obtained within a kinetic time course. Moreover, it is essential in a discontinuous assay that the solutions are stable over the time required to complete the entire assay. On the other hand, a spectroscopic assay enables a full kinetic time course to be obtained from a single experiment. This is especially advantageous for studies of multiphasic time courses since a spectroscopic assay enables many data points to be obtained within each phase of the reaction, whereas this requires multiple experiments using a discontinuous assay. The several hundred data points obtained for each unwinding trace also enables more accurate determination of the observed kinetic parameters. Furthermore, under conditions where an analytical solution of the kinetic rate equations is not possible, the data are easily imported into numerical simulation programs, such as KINSIM and FITSIM (Barshop et al., 1983; Zimmerle et al., 1987; Zimmerle & Frieden, 1989), which is required for global fitting of multiple data sets to extract individual rate constants in the kinetic mechanism.

For the specific case of helicase-catalyzed DNA unwinding, another advantage of the continuous fluorescent assay is that one can perform experiments over a much wider range of DNA substrate concentrations. Our previous chemical quench-flow studies of Rep-catalyzed DNA unwinding were performed at 1 nM DNA in order to prevent DNA reannealing both during the kinetic time course and in the time before each sample could be loaded on the gel for analysis (Amaratunga & Lohman, 1993). The fluorometric assay described here can also be performed at DNA concentrations as low as 1 nM so that reannealing occurs much slower than, and is well-separated from, DNA unwinding. However, experiments can also be performed at much higher DNA concentrations, although at the higher DNA concentrations reannealing will generally occur on the same time scale as unwinding and therefore must be considered explicitly as part of the mechanism and analyzed using simulation techniques. Such reannealing will be most important for experiments performed with excess DNA. We have determined a bimolecular rate constant of 9×10^4 M^{-1} s^{-1} for reannealing of the 18 base pair fluorescently labeled DNA substrate I under the conditions used here (data not shown). Therefore, at a single strand concentration of 1 nM, this DNA substrate will reanneal with a half-time of ~3 hours, whereas at 100 nM strands, the half-time is reduced to ~2 min. Therefore, DNA reannealing does not occur on the time scale of our experiments, which were performed at 1 nM DNA, whereas reannealing does occur on the time scale of unwinding in the experiments of Raney et al. (1994) and Houston and Kodadek (1994), which were performed at 250 and 100 nM DNA, respectively (see below).

Although fluorescence assays have numerous advantages, they will not replace chemical quench-flow studies, but rather need to be used in conjunction with such studies to verify that the spectroscopic signal change reflects DNA unwinding and to calibrate the signal change with the actual change in reactant concentration. By comparison with identical chemical quench-flow experiments, we show that the amplitude of the fluorescence change from the Rep-catalyzed stopped-flow experiment correlates well with the fraction of duplexes unwound (Figure 8) and that the stopped-flow fluorescence time courses superimpose on the DNA unwinding time courses measured by quench-flow (Figures 4B and 6). However, this agreement was obtained only after correction of the stopped-flow traces for changes in fluorescein fluorescence which do not result from changes in FRET, such as binding of the released fluorescein-labeled ss-DNA and photobleaching of the fluorescein. This correction was accomplished by subtracting the time course of the fluorescence change associated with a control experiment performed using DNA substrate II, which contains only the fluorescein donor without the hexachlorofluorescein acceptor. The comparisons in Figure 5 indicate that the kinetics of unwinding of the two DNA substrates I and II are the same within the reproducibility of the rapid quench measurement. Furthermore, since the correction due to this non-FRET fluorescence change is relatively small (cf. trace 2 vs trace 1 in Figure 4A), any differences in the unwinding kinetics for these two substrates will be within the uncertainty limits of the experiment. Indeed, the ability to isolate only fluorescence changes directly associated with the DNA unwinding process is a major advantage of the FRET assay.

In pre-steady state experiments where the helicase enzyme concentration is comparable to or greater than the DNA substrate concentration it must also be recognized that a significant fraction of the unwound ss-DNA will be bound by the helicase. Therefore, any fluorescence changes associated with protein−DNA binding must also be considered in the analysis of such experiments. For example, we have observed significant changes in fluorescence upon binding Rep to ss-oligodeoxynucleotides end-labeled with fluorescein or ss-DNA containing 2-aminopurine (K. P. Bjornson, unpublished results). Furthermore, the extent of Rep−DNA binding is linked to ATP and ADP binding (Wong & Lohman, 1992) and also to ATP hydrolysis (K. P. Bjornson, unpublished results), and this is likely to be true for other helicases as well. In the case of the DNA substrate studied here, we observe a 3-fold enhancement of fluorescein fluorescence at 520 nm upon formation of the single strands from the duplex DNA (Figure 3A). However, in the presence of Rep protein and ATP, there is only a 2-fold enhancement of fluorescence at 520 nm upon unwinding (Figure 3B). Therefore, in this case and likely for other helicases, it is not appropriate to quantitate the extent of DNA unwinding by comparison to the fluorescent signal change

observed in a DNA reannealing experiment performed in the absence of protein and ATP.

During preparation of this paper, two reports appeared describing fluorescence assays for use in studies of helicase-catalyzed DNA unwinding (Raney et al., 1994; Houston & Kodadek, 1994). Houston and Kodadek (1994) described a fluorescent DNA substrate very similar to the one described here and its use in steady state kinetic studies of DNA unwinding catalyzed by phage T4 Dda helicase. The oligodeoxynucleotide described by Houston and Kodadek (1994) has coumarin (donor), attached to the 3′ end of one DNA strand, and fluorescein (acceptor), attached to the 5′ end of the complementary strand of a 25 base pair duplex. The experiments described by these workers were performed at 100 nM DNA substrate and 10 nM Dda protein and monitored the steady state unwinding of the DNA by the increase in coumarin fluorescence. In the single turnover experiments described here, the DNA substrate I uses fluorescein as the donor and hexachlorofluorescein as the acceptor, which has three advantages over the coumarin−fluorescein pair. The first is that the maximum excitation wavelength for fluorescein (492 nm) is much higher than the maximum excitation wavelength for coumarin (385 nm). The lower excitation wavelength of coumarin overlaps with the fluorescence emission wavelength of tryptophan and thus may cause interference under certain conditions. Second, we have found that the fluorescein−hexachlorofluorescein pair is significantly more sensitive than the coumarin−fluorescein pair, thus allowing experiments to be performed at the much lower DNA concentrations (1 nM) used in our studies. Finally, both fluorescein and hexachlorofluorescein are available commercially in phosphoramidite form (see Materials and Methods) and thus can be routinely incorporated into oligodeoxynucleotides using an automated DNA synthesizer.

Raney et al. (1994) have also reported a fluorescent assay for use in studies of helicase-catalyzed DNA unwinding that utilizes oligodeoxynucleotide duplexes containing the fluorescent base 2-aminopurine (2-AP). The utility of 2-AP is that it can form a two hydrogen bonded base pair with thymidine, and furthermore, its fluorescence ($\lambda_{ex,max}$ = 310 nm; $\lambda_{em,max}$ = 365 nm) increases ~2-fold upon duplex unwinding (Bloom et al., 1993). Raney et al. (1994) showed that T4 Dda protein-catalyzed unwinding of a 28 base pair duplex containing seven 2-aminopurines could be monitored by stopped-flow fluorescence and estimated a rate constant for unwinding of ~0.4 s^{-1} (11.2 base pairs s^{-1}) at 25.0 °C. The advantage of 2-AP is that it can be positioned anywhere within a duplex, barring sequence constraints, although 2-AP was placed evenly throughout the DNA substrate used by Raney et al. (1994). On the other hand, the fluorescence change associated with 2-AP is less sensitive than that observed with the fluorescein−hexachlorofluorescein FRET pair. As a result, significantly higher DNA concentrations (250 nM) were used in the 2-AP experiments of Raney et al. (1994), and thus DNA reannealing occurred on the same time scale as DNA unwinding. Furthermore, potential changes in 2-AP fluorescence may also result from protein binding to the unwound ss-DNA. In this context, a major advantage of the FRET DNA substrate described here that is not shared by DNA substrates containing a single fluorophore (e.g., 2-AP), is that any donor fluorescence changes that do not result from FRET and thus do not directly reflect DNA unwinding can be identified through unwinding experiments performed with a DNA substrate (e.g., II) which does not contain the FRET acceptor fluorophore. In this way, one can isolate the fluorescence changes that only result from DNA unwinding.

Since the fluorescent DNA substrate described here has the FRET fluorophores attached at the end of the duplex DNA, only complete unwinding of the duplex will be monitored by the change in FRET, and thus partially unwound intermediates will not be detected. In this respect, this assay is similar to rapid chemical quench studies performed with radioactively labeled DNA (Amaratunga & Lohman, 1993). Therefore, in general, we anticipate this substrate to be most useful for pre-steady state studies of the unwinding of short duplex DNA molecules. In fact, since the Rep dimer unwinds duplex DNA by binding to the ss-DNA and duplex DNA simultaneously, one to each Rep subunit to form a P_2SD intermediate, it is likely that the 18 base pair duplex examined in this report is unwound completely in a single catalytic event (Wong & Lohman, 1992; Lohman, 1993; Amaratunga & Lohman, 1993). However, mechanistic information can also be obtained from studies of DNA substrates containing longer duplex regions even though the fluorescence signal will only detect fully unwound duplexes. For such longer DNA duplexes the time course for complete unwinding will be dependent not only upon the rate of helicase-catalyzed duplex melting, but also upon the processivity and rate of translocation of the helicase.

Mechanistic Aspects of Rep-Catalyzed DNA Unwinding. Although *E. coli* Rep protein is a stable monomer in the absence of DNA, it dimerizes upon binding DNA (Chao & Lohman, 1991; Wong et al., 1992; Wong & Lohman, 1992), and the Rep dimer appears to be the functionally active form of the helicase (Chao & Lohman, 1991; Amaratunga & Lohman, 1993). Our previous studies have suggested that Rep dimers unwind duplex DNA using an active, rolling mechanism in which Rep translocation along DNA is coupled to ATP binding, whereas DNA unwinding is coupled to ATP hydrolysis. In this model, the essential intermediate for the unwinding step is a P_2SD complex in which the Rep dimer (P_2) is bound at the ss-/ds-DNA junction to both ss-DNA and duplex DNA simultaneously, one subunit to each conformation (Wong & Lohman, 1992). Upon ATP hydrolysis, multiple base pairs within the duplex DNA bound by Rep are unwound, displacing the 5′ strand, while the Rep subunit remains bound to the 3′ strand to form a P_2S_2 complex. Release of ADP, followed by rebinding of ATP, is then coupled to Rep translocation by a rolling mechanism as one subunit releases the 3′ single strand and rebinds to the duplex (Lohman, 1992, 1993; Wong & Lohman, 1992).

Our rapid chemical quench-flow studies of Rep-catalyzed unwinding of short duplex DNA and "reverse polarity" DNA substrates support the proposal that a P_2SD complex is the productive Rep−DNA complex (Amaratunga & Lohman, 1993). The stopped-flow fluorescence experiments presented here test this proposal further by examining the sensitivity of the productive Rep−DNA complexes to challenges by competitor DNA that is added with the ATP to initiate unwinding. We show that Rep unwinds the 18 base pair duplexes containing a 3′-$(dT)_{20}$ tail in a biphasic reaction. The rate constant of the fast phase is independent of Rep concentration, $k_{obs,1}$ = 1.3 ± 0.2 s^{-1} (25.0 °C, buffer U) (Figure 7A), whereas its amplitude increases with increasing

Rep concentration (Figure 7B). Therefore, this fast phase reflects unwinding of DNA that is bound to Rep in productive complexes.[1] We also show that these productive Rep−DNA complexes are resistant to challenge by both $(dT)_{16}$ and nonfluorescent DNA (substrate III in Figure 1), even at 100-fold higher concentrations (Figure 9A). This argues strongly that both DNA binding sites of the Rep dimer are occupied in the productive complex, consistent with a P_2SD complex (Wong & Lohman, 1992).

The second, slower phase of the Rep-catalyzed DNA unwinding reaction behaves quite differently than the first phase. The observed rate constant for the second phase, $k_{obs,2}$, increases with increasing Rep concentration, while its amplitude decreases (Figure 7B,C). In addition, $k_{obs,2}$ decreases with increasing competitor DNA (Figure 9B). These results indicate that the rate of DNA unwinding in the slow phase is limited by a binding event involving Rep. From the Rep concentration dependence of $k_{obs,2}$, we calculate an apparent bimolecular rate constant of $(1.8 \pm 0.1) \times 10^5$ M^{-1} s^{-1} for Rep binding. Preliminary measurements indicate that the apparent bimolecular rate constant for Rep monomer binding to $(dT)_{16}$ under these conditions is greater than 1×10^7 M^{-1} s^{-1} (K. P. Bjornson, unpublished results); thus DNA binding is not likely to be the rate-limiting step for unwinding in the second phase. It seems more likely that the rate of DNA unwinding in the second phase is limited by the rate of Rep dimerization on the DNA substrate since Rep dimers are required to form the productive P_2SD complex (Wong & Lohman, 1992; Amaratunga & Lohman, 1993; Lohman, 1993). Furthermore, a rate constant of $(1.8 \pm 0.1) \times 10^5$ M^{-1} s^{-1} is consistent with the range of dimerization rate constants observed for proteins the size of Rep (Northrup & Erickson, 1992).

In our pre-steady state, chemical quench-flow studies of the kinetics of Rep-catalyzed DNA unwinding, we fit the biphasic unwinding time courses to an exponential "burst" phase followed by a steady state rate of unwinding (Amaratunga & Lohman, 1993), rather than the sum of two exponential phases as we do in the current study. We also reported that the second phase appeared to be independent of Rep concentration, contrary to our current conclusions based on the stopped-flow studies described here. These earlier conclusions were the result of the limited number of time points that could be sampled by the quench-flow method. This emphasizes the utility of the continuous spectroscopic method described here, which allows many more time points to be collected, thus enabling more accurate determinations of the complete time course and the observed rate constants for all phases of a multiphasic reaction.

The fluorescent unwinding assay described in this paper, in conjunction with the proven method of rapid chemical quench-flow, has enabled us to further probe the kinetic mechanism by which Rep helicase unwinds duplex DNA. A complete determination of the kinetic mechanism of Rep-catalyzed DNA unwinding will require further studies of (i) the kinetics of DNA binding to the Rep monomer and dimers, (ii) the kinetics of Rep dimerization on DNA, and (iii) the kinetics of nucleotide binding and associated conformational changes upon hydrolysis. The FRET DNA substrate described here and the 2-aminopurine substrate (Raney et al., 1994) both allow continuous monitoring of DNA unwinding by stopped-flow methods and have complementary advantages, which when combined with rapid chemical quench-flow studies (Amaratunga & Lohman, 1993) will greatly facilitate the detailed kinetic studies that are needed to understand the mechanism(s) by which helicases carry out their essential function.

ACKNOWLEDGMENT

We thank Bill van Zante for invaluable technical assistance and Janid Ali and Marilyn Ferrari for comments on the manuscript.

REFERENCES

Amaratunga, M., & Lohman, T. M (1993) *Biochemistry 32*, 6815−6820.
Barshop, B. A., Wrenn, R. F., & Frieden, C. (1983) *Anal. Biochem. 130*, 134−145.
Bloom, L. B., Otto, M. R., Beechem, J. M., & Goodman, M. F. (1993) *Biochemistry 32*, 11247−11258.
Cardullo, R. A., Agrawal, S., Flores, C., Zamecnik, P. C., & Wolf, D .E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8790−8794.
Chao, K., & Lohman, T. M. (1991) *J. Mol. Biol. 221*, 1165−1181.
Clegg, R. M. (1992) *Methods Enzymol. 211*, 353−388.
Clegg, R. M., Murchie, A. I. H., Zechel, A., & Lilley, D. M. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 2994−2998.
Cooper, J. P., & Hagerman, P. J. (1990) *Biochemistry 29*, 9261−9268.
Fairclough, R. H., & Cantor, C. R. (1978) *Methods Enzymol. 48*, 347−379.
Geider, K., & Hoffmann-Berling, H. (1981) *Annu. Rev. Biochem. 50*, 233−260.
Geiselmann, J., Wang, Y., Seifried, S. E., & von Hippel, P. H. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7754−7758.
Hill, T. L., & Tsuchiya, T. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4796−4800.
Houston, P., & Kodadek, T. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5471−5474.
Jameson, D. M. (1984) in *Fluorescein Hapten: An Immunological Probe* (Voss, E. W., Ed.) pp 23−48, CRC Press, Boca Raton, FL.
Lohman, T. M. (1992) *Mol. Microbiol. 6*, 5−14.
Lohman, T. M. (1993) *J. Biol. Chem. 268*, 2269−2272.
Lohman, T. M., Chao, K., Green, J. M., Sage, S., & Runyon, G. (1989) *J. Biol. Chem. 264*, 10139−10147.
Matson, S. W., & Kaiser-Rogers, K. A. (1990) *Annu. Rev. Biochem. 59*, 289−329.
Northrup, S. H., & Erickson, H. P. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3338−3342.
Raney, K. D., Sowers, L. C., Millar, D. P., & Benkovic, S. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 6644−6648.
Stryer, L., & Haugland, R. P. (1967) *Proc. Natl. Acad. Sci. U.S.A. 58*, 719−726.
Thommes, P., & Hubscher, U. (1992) *Chromosoma 101*, 467−473.
Wong, I., & Lohman, T. M. (1992) *Science 256*, 350−355.
Wong, I., Chao, K. L., Bujalowski, W., & Lohman, T. M. (1992) *J. Biol. Chem. 267*, 7596−7610.
Zimmerle, C. T., & Frieden, C. (1989) *Biochem. J. 258*, 381−387.
Zimmerle, C. T., Patane, K., & Frieden, C. T. (1987) *Biochemistry 26*, 6545−6552.