Escherichia coli Rep Helicase Unwinds DNA by an Active Mechanism†

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ABSTRACT: DNA helicases unwind duplex DNA to form the single-stranded (ss) DNA intermediates required for replication, recombination, and repair in reactions that require nucleoside 5′-triphosphate hydrolysis. Helicases generally require a ss-DNA flanking the duplex in order to initiate unwinding *in vitro*; however, the precise function of the ss-DNA is not understood. If a helicase unwinds DNA by a "passive" mechanism, it would bind to and translocate unidirectionally along the ss-DNA and facilitate duplex unwinding by translocating onto the ss-DNA that is formed transiently by thermal fluctuations in the duplex. We have examined the kinetics of DNA unwinding by *Escherichia coli* Rep protein (a 3′ to 5′ helicase) by rapid quench-flow methods using a series of novel, nonnatural DNA substrates possessing 3′ flanking ss-DNA within which is embedded either a segment of ss-DNA possessing reversed backbone polarity or a non-DNA [poly(ethylene glycol)] spacer, either of which should block unwinding by a passive helicase. The *E. coli* Rep helicase effectively unwinds these DNA substrates, ruling out a passive mechanism of unwinding. Instead, the results are consistent with an "active" rolling mechanism during which Rep binds to ss-DNA and duplex DNA simultaneously.

DNA helicases function in prokaryotes and eukaryotes to unwind double-stranded (ds) DNA, thus providing the single-stranded (ss) DNA intermediates required during processes such as replication, recombination, and repair (Matson & Kaiser-Rogers, 1990; Matson, 1991; Lohman, 1992, 1993; Thommes & Hubscher, 1992). These enzymes function at ss/ds-DNA junctions (e.g., unwinding forks) to catalyze DNA unwinding [breakage of the hydrogen bonds stabilizing the base pairs (bp)], some at rates of 500 to 10^3 bp/s by mechanisms that require hydrolysis of nucleoside 5′-triphosphates. In order to unwind long stretches of DNA processively, helicases must also translocate along DNA without fully dissociating.

In vitro, nearly all DNA helicases show a preference for unwinding duplex DNA possessing a ss-DNA flanking region;[1] some prefer a 3′ ss-DNA (3′ to 5′ helicases), while others prefer a 5′ ss-DNA (5′ to 3′ helicases). This ss-DNA "tail" provides a high-affinity site for initiation of unwinding; however, its precise role is not clear. In principle, helicases could utilize the flanking ss-DNA to unwind DNA by either a "passive" or an "active" mechanism (Lohman, 1992, 1993). In a passive mechanism, the helicase binds to and translocates unidirectionally, either by "sliding" or by "rolling", along the single-stranded DNA in the direction of the duplex, fueled by ATP hydrolysis (e.g., in the 3′ to 5′ direction if the helicase functions on the 3′ strand as in Figure 1A). A passive helicase would not interact with the ds-DNA but would catalyze net unwinding by binding to the ss-DNA that is formed transiently at the ss/ds-DNA junction as a result of thermal fluctuations in the DNA duplex. However, although it is often assumed that helicases can translocate with strict unidirectionality along ss-DNA alone, this has not yet been demonstrated for any helicase. Alternatively, a helicase that unwinds DNA by an active mechanism would generally bind directly to ds-DNA at the junction, as well as to ss-DNA, and destabilize some number of base pairs in each catalytic step. Therefore, most

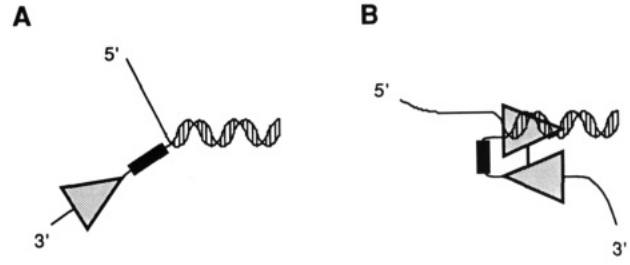

FIGURE 1: (A) Representation of a passive 3′ to 5′ helicase (triangle) that cannot unwind the duplex due to the presence of a block that can be either a segment of non-DNA or a segment of ss-DNA with reversed (5′ to 3′) polarity. (B) In an active rolling mechanism for DNA unwinding, a dimeric Rep helicase (trianglular protomers assumed to be related by C_2 symmetry) is postulated to form a P_2SD complex in which a Rep dimer is bound simultaneously to the 3′ ss-DNA and the ds-DNA (Lohman, 1992; Wong & Lohman, 1992) such that a segment of the 3′ ss-DNA containing a block can be looped out and thus circumvented.

active unwinding mechanisms require that the functionally active helicase possess at least two distinct DNA binding sites as well as the ability to bind both ss- and ds-DNA simultaneously (Lohman, 1992, 1993; Wong & Lohman, 1992). A passive helicase that translocates by a rolling mechanism would also require at least two DNA binding sites, which would bind only ss-DNA.

The *Escherichia coli* Rep protein is required for DNA replication of a number of bacteriophages (e.g., ϕX174, M13) and may be involved in chromosomal replication since *rep* mutants show a reduced rate of chromosomal fork movement (Lane & Denhardt, 1974, 1975). Rep is a 3′ to 5′ DNA helicase (Yarranton & Gefter, 1979; Lohman et al., 1989); i.e., a 3′ ss-DNA flanking the duplex greatly facilitates initiation of DNA unwinding *in vitro*. Although Rep is a monomer (M_r = 76 400)[2] in the absence of DNA (Arai et al.,

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[1] One exception is the *E. coli* RecBCD helicase which prefers to initiate unwinding at blunt-ended linear DNA (Taylor & Smith, 1985).

[2] The DNA sequence of the *rep* gene has been redetermined recently and the predicted amino acid length found to be 673 residues (Daniels et al., 1992) rather than 637 residues as first reported (Gilchrist & Denhardt, 1987). We have sequenced the pRepO plasmid, which overproduces the Rep protein (Colasanti & Denhardt, 1987) and find agreement with Daniels et al. (1992).

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1981; Lohman et al., 1989), it forms a stable dimer upon binding either ss- or ds-DNA, and the dimer appears to be the functionally active form of the Rep helicase (Chao & Lohman, 1991). Each protomer (subunit) (P) of the Rep dimer binds ss-DNA (S) and ds-DNA (D) competitively, although a Rep dimer can bind ss- and ds-DNA simultaneously to form a P_2SD complex (Wong et al., 1992). Furthermore, ADP and β,γ-imidoadenosine 5′-triphosphate [AMPP(NH)P], a nonhydrolyzable analogue of ATP, modulate Rep's DNA binding properties allosterically such that ADP binding favors formation of a Rep dimer complex in which both protomers are bound to ss-DNA (P_2S_2 complex), whereas binding of AMPP(NH)P favors formation of a P_2SD complex in which *both* ss- and ds-DNA are bound simultaneously to a Rep dimer (Wong & Lohman, 1992).

On the basis of these studies, an active rolling mechanism for DNA unwinding by Rep dimers has been proposed (Lohman, 1992; Wong & Lohman, 1992). In this model, translocation of the dimeric helicase is coupled to ATP binding and occurs by a rolling mechanism in which one Rep protomer releases the 3′ ss-DNA and subsequently binds the ds-DNA ahead of the fork, while the other protomer remains bound to the 3′ ss-DNA. Thus, a Rep dimer proceeds through two important intermediates: in one (P_2S_2 complex), both Rep protomers interact with the 3′ ss-DNA, whereas in the other (P_2SD complex), the 3′ ss-DNA and the ds-DNA are bound simultaneously as depicted in Figure 1B. While in the P_2SD complex, ATP hydrolysis is proposed to catalyze conformational changes which destabilize some number of base pairs in the duplex region, resulting in displacement of the 5′ strand while both protomers remain bound to the 3′ ss-DNA reforming the P_2S_2 complex (Lohman, 1992; Wong & Lohman, 1992). Release of ADP and inorganic phosphate and subsequent rebinding and hydrolysis of ATP catalyze successive cycles of unwinding.

To examine the role of the 3′ ss-DNA flanking region and determine whether Rep helicase unwinds DNA by an active or a passive mechanism, we have constructed a number of novel, nonnatural DNA substrates and have investigated the kinetics of Rep-catalyzed DNA unwinding using rapid chemical quench-flow techniques. The DNA substrates consist of short 18- or 24-bp duplexes possessing noncomplementary 3′ and/or 5′ ss-DNA tails. In some of the ss-DNA tails, the backbone polarity of four or five nucleotides has been reversed, and in others, a non-DNA [poly(ethylene glycol)] spacer has been inserted between the duplex and the ss-DNA tail. Either of these segments, when positioned directly adjacent to the duplex DNA, should prevent duplex DNA unwinding by Rep if it functions by a passive mechanism (see Figure 1A), whereas these segments could be looped out and thus circumvented if Rep functions by an active rolling mechanism which proceeds through a P_2SD intermediate (see Figure 1B) as previously proposed (Lohman, 1992; Wong & Lohman, 1992).

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, pH 7.5, 6 mM NaCl, 1.7 mM $MgCl_2$, 1.5 mM ATP, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol. *E. coli* Rep protein was purified to >99% homogeneity as described (Lohman et al., 1989; Chao & Lohman, 1991). Its concentration was determined spectrophotometrically, using a revised extinction coefficient[2] for the monomer of $\epsilon_{280} = 7.68 \times 10^4\ M^{-1}\ cm^{-1}$. T4 polynucleotide kinase was from U.S. Biochemical Corp. (Cleveland, OH).

Reverse Polarity and Poly(ethylene glycol) Spacer Oligodeoxynucleotides. The structures of the oligodeoxynucleotide substrates are given in Table I. The duplex regions are either 18 or 24 bp in length as indicated. The ss-DNA regions, either 3′ or 5′ tails, are composed entirely of oligodeoxythymidylates to avoid intramolecular base pairing within the ss-DNA tail and because there is no base preference for Rep binding to ss-oligodeoxynucleotides (Wong et al., 1992). A 20-nucleotide ss-DNA tail was used in these studies on the basis of the fact that a Rep monomer covers ~16 nucleotides when bound to ss-DNA (Chao & Lohman, 1991) and preliminary studies which show a near-optimum burst amplitude for substrates with 3′ ss-DNA tails of this length (M. Amaratunga, unpublished results). "Reverse polarity" substrates (**VIII**, **IX**, and **X**) contain an 18-bp duplex and $(dT)_{20}$ ss-DNA tails in which the backbone polarity of a section of the ss-DNA has been reversed. Substrate **XV** contains a $(dT)_{28}$ ss-DNA tail in which the backbone polarity of a four-nucleotide section has been reversed. "Non-DNA spacer" substrates (**XI**, **XII**, and **XIII**) contain segments of tri(ethylene glycol) $([-(CH_2)_2O-]_3)_N$ (N = 1, 3, or 4) inserted between the 3′-$(dT)_{20}$ ss-DNA tail and the 24-bp duplex.

Automated synthesis of oligodeoxynucleotides was performed with an ABI Model 391 (Applied Biosystems, Foster City, CA) using β-cyanoethyl phosphoramidite chemistry. We used 5′ cyanoethyl phosphoramidites (Glen Research, Sterling, VA) to reverse the backbone polarity within a segment of an oligodeoxynucleotide (van de Sande et al., 1988). When introduced into a normal DNA synthesis cycle, these phosphoramidites introduce a 5′ to 5′ linkage, leaving a 3′-OH group at the growing end of the DNA. After a given number of cycles in the reverse polarity, the original polarity of the DNA backbone can be reintroduced by using the conventional β-cyanoethyl phosphoramidite, which introduces a 3′–3′ linkage and a free 5′-OH group. Non-DNA [tri(ethylene glycol)] linkers were incorporated into oligodeoxynucleotides by using dimethoxytrityltri(ethylene glycol) 2-cyanoethyl *N,N*-diisopropylphosphoramidites (Glen Research, Sterling, VA). In this way, one or more $([-(CH_2)_2O-]_3O$ moieties can be introduced into the DNA (Durand et al., 1990). Oligodeoxynucleotides were deblocked and purified (>99%) by electroelution of DNA from denaturing polyacrylamide gels as described (Wong et al., 1992). DNA concentrations were determined spectrophotometrically. Some oligodeoxynucleotides were radiolabeled with ^{32}P on the 5′ end as described (Wong et al., 1992). Double-stranded DNA was prepared by mixing equal moles of 5′-^{32}P-labeled DNA and the complementary unlabeled strand (0.2 μM strands) in 20 mM Tris (pH 7.0), 50 mM NaCl, and 1 mM $MgCl_2$, followed by heating for 3 min at 90 °C and slow cooling 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). All reactions were performed at 25.0 °C in buffer U. Rep protein (typically 1 μM monomer) and DNA substrate (2 nM) (^{32}P 5′-end-labeled on one strand) were premixed in 2× buffer U without ATP for 10 min at 4 °C (incubations of up to 1 h gave identical results) and loaded in one loop (45 μL). The second loop (45 μL) contained 3 mM ATP in H_2O (pH 7). The samples were then incubated in the loops for 4 min at 25 °C (longer preincubation times up to 8 min gave identical results). 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 80 s. Final DNA concentrations of 1 nM were used in order to prevent renaturation of the DNA

Table I: Kinetic Parameters for Rep-Catalyzed Unwinding of DNA[a]

	DNA substrate	A_b	k_b (s^{-1})	$V_{ss} \times 10^2$ (s^{-1})
I	$_{3'}$⊓18⊓$_{5'}$.[b]			0.12 (±0.01)
II	$_{3'}$⊓24⊓$_{5'}$.[c]			0.16 (±0.02)
III	3'-(T_{20})⊓18⊓$_{5'}$	0.77 (±0.06)	0.16 (±0.02)	0.23 (±0.09)
IV	3'-(T_{20})⊓24⊓$_{5'}$	0.75 (±0.05)	0.17 (±0.02)	0.24 (±0.08)
V	5'-(T_{20})⊓18⊓$_{5'}$	0.07 (±0.01)	0.10 (±0.01)	0.14 (±0.01)
VI	5'-(T_{20})⊓24⊓$_{5'}$			0.16 (±0.01)
VII	5'-(T_{20}) 3'-(T_{20})⊓24⊓$_{5'}$	0.53 (±0.05)	0.15 (±0.02)	0.39 (±0.09)
VIII	3'-(T_{16})-5'-5'-(T_4)-3'-3⊓18⊓$_{5'}$	0.68 (±0.04)	0.08 (±0.01)	0.24 (±0.06)
IX	3'-(T_{12})-5'-5'-(T_4)-3'-3'-(T_4)⊓18⊓$_{5'}$	0.44 (±0.03)	0.09 (±0.01)	0.37 (±0.05)
X	3'-(T)-5'-5'-(T_{14})-3'-3'-(T_5)⊓18⊓$_{5'}$	0.04 (±0.01)	0.17 (±0.04)	0.19 (±0.01)
XI	3'-(T_{20})-{[(CH_2)$_2$-O-]$_3$}-(T)⊓24⊓$_{5'}$	0.56 (±0.05)	0.06 (±0.01)	0.32 (±0.07)
XII	3'-(T_{20})-{[(CH_2)$_2$-O-]$_3$}$_3$-(T)⊓24⊓$_{5'}$	0.31 (±0.05)	0.06 (±0.01)	0.41 (±0.06)
XIII	3'-(T_{20})-{[(CH_2)$_2$-O-]$_3$}$_4$-(T)⊓24⊓$_{5'}$	0.21 (±0.04)	0.07 (±0.02)	0.40 (±0.06)
XIV	3'-(T)-{[(CH_2)$_2$-O-]$_3$}$_4$-(T)⊓24⊓$_{5'}$			0.18 (±0.02)
XV	3'-(T_{20})-5'-5'-(T_4)-3'-3'-(T_4)⊓18⊓$_{5'}$	0.58 (±0.03)	0.09 (±0.01)	0.30 (±0.06)

[a] All reactions were performed at 25.0 °C in 20 mM Tris, pH 7.5, 6 mM NaCl, 1.7 mM $MgCl_2$, 1.5 mM ATP, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol. The kinetic parameters were obtained by nonlinear least squares analysis (Johnson & Frasier, 1985) of the data according to eq 1; the reported error limits represent 67% confidence limits. [b] 18-bp duplex top-strand sequence: 5'GCCTCGCTGCCGTCGCCA3'. [c] 24-bp duplex top-strand sequence: 5'GCCCTGCTGCCGACCAACGAAGGT3'.

after quenching with SDS. The final concentration of Rep during each reaction was 500 nM (monomer), except for data in Figure 2B, where it varied as indicated. The quenched samples were subjected to polyacrylamide gel electrophoresis in order to separate the ^{32}P duplex DNA from the unwound ^{32}P ss-DNA (20% PAGE for 18-bp duplexes and 15% PAGE for 24-bp duplexes). 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, $[S(t)]/[D_T]$, was calculated as

$$[S(t)]/[D_T] = ([C_s(t)/(C_s(t) + C_D(t))] - [C_{s,0}/(C_{s,0} + C_{D,0})])/(1 - [C_{s,0}/(C_{s,0} + C_{D,0})])$$

where $C_s(t)$ and $C_D(t)$ are the radioactive counts within each band corresponding to single-stranded and duplex DNA, respectively, at time t. $C_{s,0}$ and $C_{D,0}$ are the corresponding quantities at $t = 0$. The amount of unwound DNA at $t = 0$ never exceeded 5%.

RESULTS

Single Turnover Kinetics of Rep-Catalyzed DNA Unwinding. Single turnover kinetics of Rep-catalyzed DNA unwinding were performed with a rapid chemical quench apparatus at 25.0 °C in buffer U using a large excess of Rep (generally 500 nM monomer) over DNA substrate (1 nM). Rep protein was premixed with DNA, and reactions were initiated by addition of 1.5 mM ATP and quenched after time t by addition of SDS. The fraction of duplexes unwound at each time was determined after separation of the duplex from the unwound ss-DNA by polyacrylamide gel electrophoresis as shown in Figure 2A.

Figure 2B shows the results of a series of kinetic experiments performed at several Rep concentrations (25 nM to 1 μM monomer), all in excess over the [DNA] (1 nM), using DNA substrate **IV** consisting of a 24-bp duplex and a 3'-$(dT)_{20}$ flanking ss-DNA. At each Rep concentration, the time–course of DNA unwinding is biphasic and can be described by a pre-steady-state "burst" phase followed by a steady-state phase as

$$[S(t)]/[D_T] = A_b(1 - \exp(-k_b t)) + v_{ss}t \quad (1)$$

where k_b is the rate constant for the burst phase, A_b is the amplitude of the burst phase, v_{ss} is the steady-state velocity of unwinding, and $[S(t)]/[D_T]$ is the fraction of duplexes

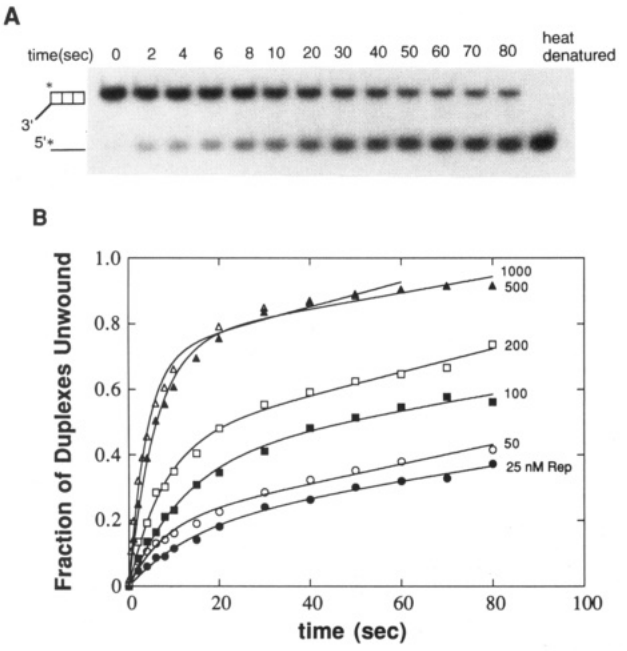

FIGURE 2: (A) Autoradiograph of a 20% polyacrylamide gel after electrophoresis of samples from a rapid quench kinetics study of Rep-catalyzed unwinding of DNA substrate **III**. The number of seconds after addition of ATP to a preformed Rep–DNA complex is indicated above each lane. Experiments were carried out at 25.0 °C in buffer U, 1 nM DNA, and 50 nM Rep. (B) Biphasic time courses of Rep-catalyzed unwinding of DNA substrate **IV** [24-bp duplex with a 3′-$(dT)_{20}$ flanking ss-DNA]. Rapid chemical quench experiments were performed at 25.0 °C in buffer U, using 1 nM DNA and the indicated Rep (monomer) concentrations. The fraction of duplex molecules unwound is plotted as a function of time.

unwound at time t. Each curve in Figure 2B was analyzed by nonlinear least squares methods (Johnson & Frasier, 1985) to obtain the three parameters, A_b, k_b, and v_{ss}. The results indicate that $k_b = 0.17 \pm 0.02\ s^{-1}$ and $v_{ss} = 0.003 \pm 0.001$ nM duplex s^{-1}, independent of Rep concentration; however, the amplitude of the burst phase, A_b, increases with increasing Rep concentration until ~500 nM Rep. The rate parameters and amplitudes of the kinetic profiles obtained at 500 nM Rep are listed in Table I for all of the DNA substrates examined. The results of these experiments are highly reproducible, as indicated by the data in Figure 4A for two independent preparations of substrate **III**. No unwinding was observed in the absence of ATP or upon substitution of the nonhydrolyzable analogue AMPP(NH)P.

Due to the presence of excess Rep, the pre-steady-state burst phase reflects single turnover unwinding of DNA substrates that were bound to Rep in productive complexes at the time of addition of ATP. This is consistent with the fact that the rate constant, k_b, is independent of [Rep] and that the amplitude, A_b, increases with increasing [Rep], reflecting an increase in the fraction of DNA bound in productive initiation complexes. The need for a large excess of Rep to obtain a significant burst phase and the fact that the burst amplitude does not reach a plateau value until ~500 nM Rep are consistent with the following known DNA binding properties of Rep (Wong & Lohman, 1992; Wong et al., 1992): (i) Rep is monomeric in the absence of DNA and dimerizes only upon binding DNA with a dimerization constant $\sim 2 \times 10^8\ M^{-1}$, and (ii) the binding affinity of Rep monomers for DNA is only $\sim 10^6\ M^{-1}$ under these conditions. Since the Rep dimer appears to be the functionally active form of the Rep helicase (Chao & Lohman, 1991; Wong & Lohman, 1992), then formation of a productive initiation complex requires these high Rep concentrations.[3] The fact that the

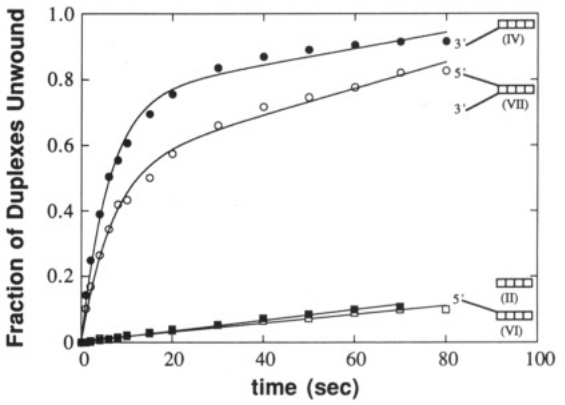

FIGURE 3: Rep-catalyzed DNA unwinding of 24-bp DNA duplexes, with or without $(dT)_{20}$ ss-DNA flanking regions, was examined at 25.0 °C in buffer U, using 1 nM DNA and 500 nM Rep (monomer). The DNA substrates are (□) **II**, (●) **IV**, (■) **VI**, and (○) **VII** (see Table I).

burst amplitude reaches a maximum of only ~0.8 suggests that 20% of the DNA is bound to Rep in nonproductive complexes, even at saturating [Rep]. In the following studies with different DNA substrates, we show only data obtained with 500 nM Rep.

Formation of Productive Rep–DNA Complexes Requires a 3′ ss-DNA Flanking Region but Not a 5′ ss-DNA Flanking Region. Rep-catalyzed unwinding of four DNA substrates (**II**, **IV**, **VI**, and **VII**) was examined to determine the effect of a 5′ flanking ss-DNA on a substrate with a 3′ flanking ss-DNA. Each DNA in Figure 3 possesses the same 24-bp duplex and each oligo(dT) tail is 20 nucleotides (see Table I for duplex sequence). No burst phase of unwinding is observed for either the fully blunt-ended substrate **II** or substrate **VI**, which possesses a 5′-$(dT)_{20}$ tail, indicating that Rep does not form productive initiation complexes with either substrate. These substrates are unwound to a small degree (~10% after 80 s), which probably reflects a slow rate of initiation of unwinding at blunt ends. However, both substrate **IV**, containing a 3′-$(dT)_{20}$ tail, and the forked substrate **VII**, containing both 3′- and 5′-$(dT)_{20}$ tails, show significant burst phases with identical rates of unwinding (see Table I). These results indicate that only a 3′ ss-DNA tail is needed for Rep to form a productive initiation complex with a 24-bp duplex DNA; a 5′ ss-DNA tail is neither required nor does it enhance initiation or the rate of unwinding of a 24-bp duplex. In fact, a significantly lower burst amplitude is observed for unwinding of the forked DNA substrate **VII**, indicating that the 5′ ss-DNA tail inhibits formation of productive Rep–DNA complexes on the 3′ ss-DNA, probably due to formation of alternative nonproductive complexes. However, the presence of the 5′-$(dT)_{20}$ tail does not affect k_b, the rate constant for DNA unwinding in the burst phase (see Table I). These results rule out any mechanisms that require Rep to interact with the 5′ ss-DNA tail.

DNA Substrates Possessing 3′ ss-DNA Tails Containing Sections with Reversed Backbone Polarity or Non-DNA Segments Can Be Unwound by Rep. In order to test whether Rep helicase uses a passive unwinding mechanism, we constructed a number of nonnatural DNA substrates with 3′ ss-DNA tails containing segments that should block unwinding

[3] Consistent with the requirement for a large excess of Rep in these kinetic studies, we have observed by nitrocellulose filter binding with DNA molecules similar to substrate **IV** (performed at 100 nM DNA) that ~50% of the DNA is bound at ~200 nM Rep and ~95% of the DNA is bound at ~500 nM Rep (M. Amaratunga, unpublished data).

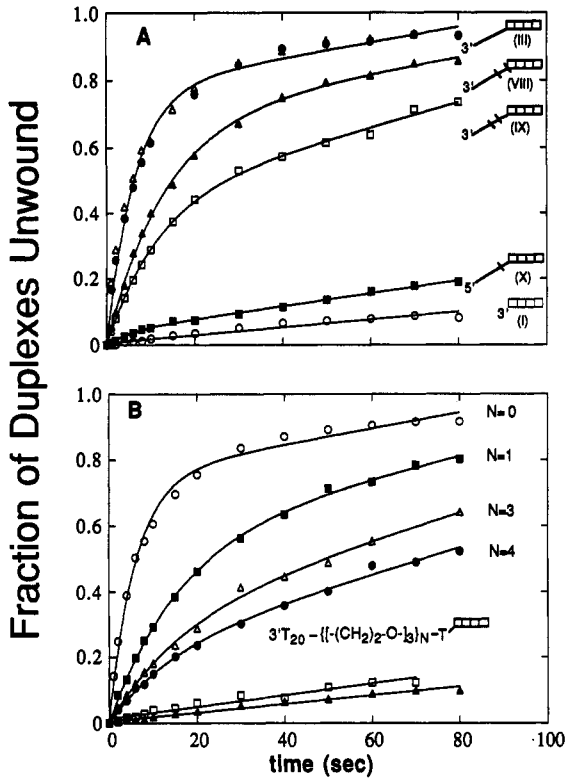

FIGURE 4: Rep-catalyzed unwinding of DNA containing either a reversal of backbone polarity or a poly(ethylene glycol) spacer within the 3′ ss-DNA tail. Experiments were performed at 25.0 °C in buffer U, using 1 nM DNA and 500 nM Rep (monomer). (A) Reverse polarity DNA substrates contain an 18-bp duplex region with ss-DNA flanking regions containing segments of mixed polarity: (○) **I**, (●, △) **III**, (▲) **VIII**, (□) **IX**, and (■) **X** (see Table I). (B) PEG spacer DNA substrates contain a 24-bp duplex with N tri(ethylene glycol) spacers inserted between the duplex and a 3′-$(dT)_{20}$ ss-DNA flanking region: (○) **IV**, (■) **XI** (N = 1), (△) **XII** (N = 3), (●) **XIII** (N = 4), (□) **XIV**, and (▲) **II** (blunt-ended duplex) (see Table I).

by a passive helicase that translocates either by sliding or by rolling unidirectionally along ss-DNA in a 3′ to 5′ direction. In Figure 4A we show the kinetics of Rep-catalyzed unwinding of two 18-bp reverse polarity substrates (**VIII** and **IX**) that contain a stretch of four nucleotides with 5′ to 3′ polarity embedded within the 3′ ss-DNA tail. Rep unwinds both of these reverse polarity substrates with significant burst amplitudes, although with rate constants, k_b, that are approximately a factor of 2 lower (0.08–0.09 s^{-1}) than for unwinding the same 18-bp duplex possessing a normal 3′-$(dT)_{20}$ tail (**III**). The amplitudes of the burst phases for the two reverse polarity substrates are also lower than for substrate **III**, which may reflect the ability of Rep to form additional nonproductive complexes with substrates **VIII** and **IX**. However, the lower amplitudes also partly reflect the progressive shortening of the end region of the ss-DNA tail possessing the correct 3′ to 5′ backbone polarity from 20 to 16 to 12 nucleotides in proceeding from substrates **III** to **VIII** to **IX**. This is clear from the fact that unwinding of DNA substrate **XV**, which is identical to substrate **IX** except that the 3′-$(dT)_{12}$ tail has been lengthened to a 3′-$(dT)_{20}$ tail, shows an increased amplitude with no change in k_b (see Table I). Rep unwinds only slightly a fully blunt-ended 18-bp duplex (**I**) as well as substrate **X**, which has a 5′-$(dT)_{15}$ ss-DNA joined by a 3′–3′ linkage to a 3′ to 5′ oriented $(dT)_5$ tail. Comparison of the Rep-catalyzed unwinding of substrates **IX**, **XV**, and **X** shows clearly that the backbone polarity of the ss-DNA region that is *eight* nucleotides removed from the ss/ds-DNA junction has a dramatic effect on the ability of Rep to form a productive initiation complex.

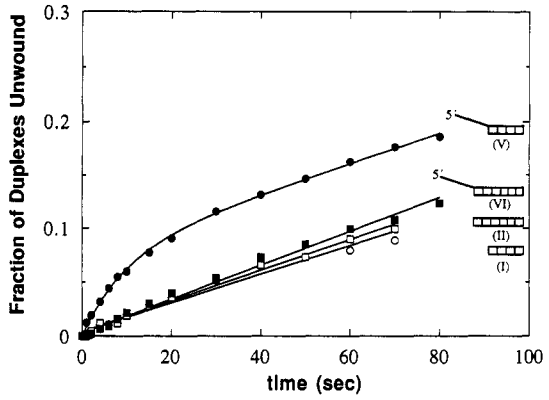

FIGURE 5: Rep-catalyzed unwinding of 18- and 24-bp DNA duplexes, with and without a 5′-$(dT)_{20}$ ss-DNA flanking region, was examined at 25.0 °C in buffer U, using 1 nM DNA and 500 nM Rep (monomer). The DNA substrates are (●) **V**, 18-bp duplex + 5′-$(dT)_{20}$ tail; (■) **VI**, 24-bp duplex + 5′-$(dT)_{20}$ tail; (○) **I**, 18-bp blunt-ended duplex; (□) **II**, 24-bp blunt-ended duplex (see Table I).

As a further test of a passive mechanism, we examined Rep's ability to unwind DNA substrates containing tri(ethylene glycol) ($[-(CH_2)_2O-]_3$)$_N$ segments inserted between a 3′-$(dT)_{20}$ ss-DNA tail and a 24-bp duplex. If the Rep helicase unwinds passively by translocating unidirectionally along the 3′-$(dT)_{20}$ ss-DNA tail, then the poly(ethylene glycol) spacers should block translocation and thus prevent unwinding of this DNA substrate. We have investigated three such substrates, **XI**, **XII**, and **XIII**, with N = 1, 3, and 4 tri(ethylene glycol) spacers, respectively. As shown in Figure 4B, Rep unwinds all three substrates with significant burst phases. The rate constants, k_b, for the burst phase for all three substrates are identical (k_b = 0.06–0.07 s^{-1}), although a factor of ~2 lower than k_b for the standard 3′-$(dT)_{20}$ ss-DNA tailed duplex with no spacer. The amplitude of the burst phase decreases with increasing number of tri(ethylene glycol) spacers (see Table I), which may reflect the lower probability of forming a looped P_2SD complex (see Figure 1B) as the spacer length between the duplex and ss-DNA regions increases (Shore et al., 1981). A control DNA substrate (**XIV**), containing the 24-bp duplex with a 3′-T-($[-(CH_2)_2O-]_{12}$ tail, shows no burst phase, with unwinding kinetics that are the same as those for the blunt-ended duplex or the duplex with a 5′-$(dT)_{20}$ flanking ss-DNA. Clearly, the 3′-$(dT)_{20}$ flanking ss-DNA is essential for Rep to form a productive initiation complex but does not need to be contiguous to the duplex in order to function.

Although a 5′ ss-DNA flanking region does not facilitate unwinding of a 24-bp duplex by Rep, Figure 5 shows that a shorter 18-bp duplex possessing only a 5′-T_{20} ss-DNA flanking region (substrate **V**) can be unwound with a small, but significant (~7%) burst amplitude (see also Table I). This result definitely rules out a strictly passive unwinding mechanism, since if Rep translocated along ss-DNA unidirectionally in a 3′ to 5′ direction, then no burst phase should be observed with substrate **V** since Rep would bind to the 5′ strand and move *away* from the duplex region.

DISCUSSION

The results reported here demonstrate that Rep helicase does *not* unwind DNA by a passive mechanism. Furthermore, they rule out any mechanisms that require interaction of Rep with the 5′ ss-DNA tail at the unwinding fork. Although Rep-catalyzed DNA unwinding requires a correctly oriented 3′ ss-DNA flanking the duplex, our results indicate that the 3′ ss-DNA need not be directly contiguous with the ds-DNA at the ss/ds-DNA junction. This rules out any mechanism

in which Rep slides into the duplex from the adjacent single-stranded DNA and also indicates that a single Rep protomer (subunit) does not recognize the ss/ds-DNA junction (cf. substrates **VIII** and **IX** in Figure 4A).

A helicase that functions by a passive rolling mechanism, as opposed to a passive sliding mechanism, could bypass the "blocks" used in our studies if a large enough region of ss-DNA were available for binding on the other side of the block. However, in the reverse polarity substrate (**VIII**), there is no intervening ss-DNA of the correct polarity between the duplex region and the block, and all of the "PEG spacer" DNA substrates have only a single T residue between the duplex and the block. In order for Rep to unwind such substrates by a passive rolling mechanism, significantly more than 1 or 2 base pairs in the duplex region (more like ~5–10 bp) would have to unwind transiently in order to provide a ss-DNA region large enough to accommodate binding of a Rep subunit across the block. Such an event would have a very low probability of occurrence. In addition, substrates **IX** and **XV** are unwound with the same value of k_b but *lower* amplitudes than substrate **VIII**; however, if a passive rolling mechanism were in operation, it would be expected that substrates **IX** and **XV** should be unwound at a faster rate due to the presence of the correctly oriented 3′-T_4 ss-DNA region adjacent to the duplex.

Further evidence that Rep does not unwind DNA by a passive mechanism (either sliding or rolling) is based on the results in Figure 5 which show a burst phase for Rep-catalyzed unwinding of an 18-bp duplex possessing only a 5′ ss-T_{20} tail (substrate **V**). A passive "3′ to 5′" helicase could not possibly unwind a duplex possessing only a 5′ ss-DNA flanking region, since it would translocate away from the duplex. Therefore, the results in Figure 5 also indicate that Rep must not translocate unidirectionally along ss-DNA. Rather, the observed 3′ to 5′ unwinding "polarity" likely results when the Rep dimer is presented with an asymmetric decision at a ss/ds-DNA junction as previously proposed (Lohman, 1992, 1993; Wong & Lohman, 1992).

Instead, all of these results are consistent with an active mechanism in which a dimeric Rep helicase unwinds DNA by binding directly to the duplex and translocates by rolling as proposed (Lohman, 1992; Wong & Lohman, 1992). During an essential intermediate step in this rolling mechanism, a P_2SD complex is formed upon ATP binding in which a Rep dimer interacts simultaneously with both ds-DNA and a region of the 3′ ss-DNA. In such an intermediate, any non-DNA or ss-DNA segment with reversed backbone polarity can be looped out, as depicted in Figure 1B, so that it does not interact with the helicase dimer. In this manner, such segments could be circumvented by the functional Rep dimer. The reduction in burst amplitude, with no change in k_b, upon increasing the length of the PEG spacer in substrates **XI**, **XII**, and **XIII** (Figure 4B) is also consistent with a looped P_2SD complex (Shore et al., 1981).

The observation of a burst phase with substrate **V** (see Figure 5) can also be explained by an active rolling model if a P_2SD intermediate of the type shown in Figure 1B is formed but using the 5′ ss-DNA tail. An explanation for why an 18-bp, but not a 24-bp, duplex can be unwound is as follows. Rep, when normally bound in a P_2SD complex with the 3′ ss-DNA tail, may unwind the duplex by "peeling" away the complementary strand of the duplex *starting from the ss/ds-DNA junction*. Assuming that a Rep protomer binds to ss-DNA with polarity, then when the Rep dimer is bound in a P_2SD complex with the 5′ ss-DNA tail, Rep would have to "peel" away the complementary duplex strand starting from the nonjunction end of the duplex. If the duplex is short enough (e.g., 18 bp), the blunt end of the DNA may be positioned within Rep's active site, thus enabling some fraction of these duplexes to be unwound, whereas the blunt end of a longer duplex (24 bp) may be positioned outside of Rep's active site and hence could not be unwound.

Although the *E. coli* Rep helicase unwinds DNA by an active mechanism, it is certainly possible that some helicases may function by passive mechanisms. As noted previously (Lohman, 1992, 1993), many (possibly all) DNA helicases appear to function as oligomeric structures, generally dimers or hexamers. A major function of these oligomeric structures may be to provide the functional helicase with multiple DNA binding sites that are a necessary feature of any active mechanism for DNA unwinding such as the rolling or inchworm model (Lohman, 1992, 1993), although a passive rolling mechanism would also require multiple DNA binding sites. Use of the novel DNA substrates described here should facilitate the determination of whether other helicases function by active or passive mechanisms.

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