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Escherichia coli helicase II (UvrD) protein initiates DNA unwinding at nicks and blunt ends

(DNA repair/helicase/electron microscopy)

GREGORY T. RUNYON*, DAVID G. BEAR†, AND TIMOTHY M. LOHMAN*‡

*Department of Biochemistry and Biophysics, Texas A&M University, College Station, TX 77843-2128; and †Department of Cell Biology and the Cancer Center, University of New Mexico School of Medicine, Albuquerque, NM 87131

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ABSTRACT **The *Escherichia coli* uvrD gene product, helicase II, is required for both methyl-directed mismatch and *uvrABC* excision repair and is believed to function by unwinding duplex DNA. Initiation of unwinding may occur specifically at either a mismatch or a nick, although no direct evidence for this has previously been reported. It has recently been shown that helicase II can unwind fully duplex linear and nicked circular DNA with lengths of at least ≈2700 base pairs *in vitro*; hence, a flanking region of single-stranded DNA is not required to initiate DNA unwinding. In studies with uniquely nicked duplex DNA, we present EM evidence that helicase II protein initiates DNA unwinding at the nick, with unwinding proceeding bidirectionally. We also show that helicase II protein initiates DNA unwinding at the blunt ends of linear DNA, rather than in internal regions. These data provide direct evidence that helicase II protein can initiate unwinding of duplex DNA at a nick, in the absence of auxiliary proteins. We propose that helicase II may initiate unwinding from a nick in a number of DNA repair processes.**

The *Escherichia coli* helicase II protein, the product of the *uvrD* gene (1–4), is a DNA-dependent ATPase and helicase (5, 6), which catalyzes the unwinding of duplex DNA with an apparent 3′ to 5′ polarity (7). Helicase II has been implicated to function in replication (8), as well as recombination (9–11). This protein is also required in methyl-directed mismatch (12–14) and *uvrABC* excision repair pathways (15–17), both of which require a nicked DNA as an intermediate. In methyl-directed mismatch repair, helicase II protein has been proposed to initiate DNA unwinding at the mismatch (18), although initiation at the nick has not been ruled out. Most previous studies of DNA unwinding by helicase II *in vitro* have concluded that a flanking region of single-stranded DNA (ssDNA), 3′ to the duplex, is required to initiate unwinding (7, 19). These apparently inconsistent observations could be reconciled if other protein factors can stimulate helicase II to initiate unwinding at a nick or a mismatch. However, Runyon and Lohman (20) have recently shown that *E. coli* helicase II can completely unwind long stretches [2700 base pairs (bp)] of fully duplex DNA *in vitro*, in the absence of flanking ssDNA, when the DNA possesses either blunt ends or a nick, although the pathway for initiation of the unwinding event was not determined. The blunt end or nick serves either as an entry point for initiation of unwinding by helicase II or possibly to relieve a topological constraint that prevents the unwinding of covalently closed circular DNA. In this report, we present EM evidence that helicase II protein can initiate unwinding of duplex DNA specifically at both a nick and a blunt end. This fact suggests that a preformed region of ssDNA may not be required for helicase II-initiated unwinding of duplex DNA *in vivo*.

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MATERIALS AND METHODS

Buffers and Enzymes. Buffers were made with distilled deionized Milli-Q water or, for EM, two times glass-distilled water that was redistilled within 12 hr of use. TE buffer is 10 mM Tris·HCl (pH 8.1)/1 mM Na_3EDTA. Unwinding buffer (pH 7.5 at 37°C) is 40 mM Hepes·KOH/0.1 mM dithiothreitol/1.5 mM ATP/0.5 mM $MgCl_2$.

Helicase II protein was purified from *E. coli* N4830/pTL51, and its concentration was determined spectrophotometrically (20). Helicase II protein stocks were tested for exonuclease activity on both 5′ and 3′ end-labeled ss- and duplex DNA under the same conditions used in the unwinding experiments. Helicase II and ^{32}P end-labeled DNA were incubated at 37°C and then subjected to electrophoresis on polyacrylamide gels. After 45 min, <0.4% of the ^{32}P label had been removed from the ends of the DNA substrates. f1 gene II protein was purified from *E. coli* K561/pDG117IIA (21), according to S. Johnston and D. Ray (University of California at Los Angeles) with some modifications.

DNA Substrates. M13mp11 replicative form (RF) DNA and pUC8 DNA were isolated as described (20); DNA concentrations were measured spectrophotometrically in TE buffer [ε_{260} = 6500 M^{-1} (nucleotide) cm^{-1}]. Blunt-ended pUC8 and M13mp11 RF DNA were formed by treatment with *Sma* I restriction enzyme. Circular M13mp11 RF DNA with a single, unique nick in the + strand was prepared by treatment of supercoiled DNA with f1 gene II protein as described (22). Greater than 90% of the RF DNA was converted to the nicked form. The reaction was stopped with 10 mM Na_3EDTA, and the DNA was dialyzed versus TE buffer/0.2 M NaCl, extracted with phenol (see below), and resuspended in TE buffer. The integrity of the nicked DNA was checked by treatment with *Hae* III restriction enzyme before and after nicking with gene II protein, followed by 5′ end-labeling of the DNA and electrophoresis under alkaline conditions. The appearance of a distinct 56-nucleotide fragment in the samples treated with f1 gene II protein indicated that a single, unique nick had been made at the expected site, thus ruling out the formation of a gap. The nicked, blunt-ended DNA substrate was prepared by linearizing the f1 gene II-nicked M13mp11 DNA by treatment with *Sna*BI. In this molecule, the nick is located on the + strand (21), 2731 bp from its 5′ end, yielding a "short arm" (2731 bp, 37%) and a "long arm" (4513 bp, 63%) duplex on either side of the nick (Fig. 3*A*). DNA samples were extracted with (*i*) phenol, (*ii*) phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol/vol), (*iii*) chloroform/isoamyl alcohol (24:1, vol/vol), precipitated with 70% ethanol/0.1 M NaCl, washed with 70% (vol/vol) ethanol, dried, and resuspended in TE buffer.

Electron Microscopy. DNA unwinding reactions (100 μl) containing 0.25 μM helicase II (monomer) and 15 μM DNA

Abbreviations: ssDNA, single-stranded DNA; RF, replicative form.

‡To whom reprint requests should be addressed at: Biochemistry and Molecular Biophysics, Washington University School of Medicine, Box 8231, 660 South Euclid Avenue, Saint Louis, MO 63110.

(nucleotide) in unwinding buffer (without Mg^{2+}) were preincubated for 2 min at 37°C, and unwinding was initiated by adding $MgCl_2$ to a final concentration of 0.5 mM [44 mM (Na^+ plus K^+)]. Reactions proceeded at 37°C for 15 sec to 5 min and were terminated by addition of Na_3EDTA to a final concentration of 12.5 mM. Fixation was carried out by the addition of 10 μl of 10% (vol/vol) formaldehyde (depolymerized by heating) and 10 μl of 6% (vol/vol) glutaraldehyde (Sigma EM grade; made by diluting a 25% stock with 40 mM Hepes·KOH/0.1 mM dithiothreitol) and then incubated for 10 min at 4°C. The fixed complexes were filtered on a 2-ml column of Sepharose CL-4B (Pharmacia) equilibrated in TE buffer. The void volume was collected (400 μl) and used for mounting on 400-mesh carbon-coated grids as described (23). The grids were rotary shadowed with 35–50 Å of tungsten at an angle of 7.1 degrees and imaged in a Hitachi H-600 EM at 50 kV. Photographs were taken at magnifications of 17,000–60,000 (Kodak 4489 EM film).

DNA Contour Lengths. The locations of internal sites of unwinding on the uniquely nicked, linear M13mp11 DNA were mapped by measuring the length of one or both of the duplex arms (Fig. 3*A*). DNA contour lengths were measured from photographs at magnifications of 100,000–350,000×, by using a Zeiss/Kontron digitizing tablet. Lengths of partially unwound molecules were measured on both the duplex and one of the helicase II-coated ssDNA and are reported as fractions of full-length duplex DNA. These measured lengths are minimum estimates for two reasons: (*i*) helicase II-coated ssDNA is compacted to an unknown and variable degree, and (*ii*) the internally unwound DNA regions were not included because they were generally too compacted to trace. To minimize these uncertainties, measurements were made only on DNA molecules that had a total measured length of ≥≈80% of the full duplex length.

RESULTS

Helicase II Initiates Unwinding from the Ends of Blunt-Ended DNA. Our initial experiments tested whether helicase II initiates unwinding from the ends or internal regions of fully duplex DNA. In these studies, we mixed fully duplex (blunt-ended) linear pUC8 DNA (2671 bp) with helicase II protein at a ratio of 0.017 helicase II (monomer)/nucleotide and visualized these by EM as a function of time after initiation of unwinding. Fig. 1 shows a series of helicase II–DNA complexes at various stages of DNA unwinding. The regions of unwound ssDNA are coated with helicase II protein; hence, they appear thicker than the duplex DNA. Fig. 1 *A* and *B* show a series of molecules at the earliest times examined (15 sec). In each case, the helicase II protein is bound exclusively to the ends of these blunt-ended DNA molecules. At least one of the molecules in Fig. 1*A* shows the beginnings of a tailed structure on one end, coated with helicase II protein, which we interpret as the initiation of DNA unwinding. Under the conditions of these experiments, at 15 sec, most of the DNA molecules did not have helicase II protein bound, and no DNA molecules were completely unwound. The binding of helicase II to the blunt ends of the DNA was only seen in the presence of both ATP and Mg^{2+} (data not shown).

Fig. 1 *C* and *D* show molecules 30 sec after the start of the unwinding reaction. At this time, the majority of the DNA molecules showed initiation of unwinding from at least one end, although most were not significantly unwound; however, complete unwinding of several DNA molecules was seen. At the 30-sec time point, we analyzed the frequency with which unwinding occurred from either the ends or internal regions on these DNA molecules (see Table 1). Only intact DNA molecules showing <≈10% unwinding were used for this analysis. Of 406 DNA molecules counted, 63% of the DNA ends had helicase II protein bound or were partially unwound. No initiation of unwinding was seen from internal regions of the DNA. The appearance of isolated binding of helicase II to internal regions of duplex DNA was seen on 13.6% of these molecules (e.g., see Fig. 1*B*); however, internally bound protein never had the filamentous appearance associated with unwound regions of DNA and occurred at

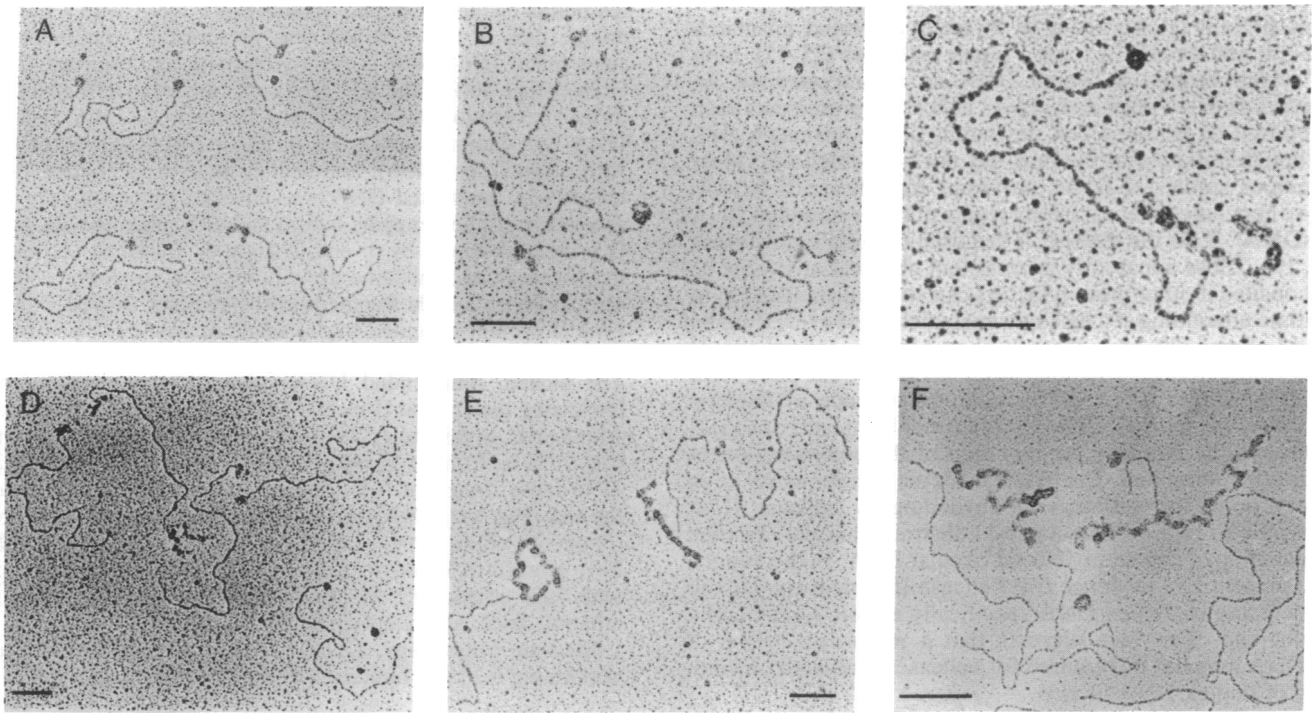

FIG. 1. Electron micrographs of blunt-ended pUC8 DNA after incubation with helicase II in unwinding buffer for various times at 37°C (0.017 helicase II monomer per nucleotide). (*A*) 15 sec. (*B*) 15 sec. (*C*) 30 sec. (*D*) 30 sec. (*E*) 3 min. (*F*) 5 min. Helicase II-coated ssDNA appears thicker than duplex DNA. (Bars = 0.1 μm.)

Table 1. Initiation of unwinding on blunt-ended pUC8 DNA

Type of complex	DNA molecules,* no.
Unwinding from internal sites	0
Unwinding from one end	233
Unwinding from both ends	141
Protein bound at internal sites	55
No unwinding or internal binding	26

*A total of 406 molecules were analyzed.

random sites. It is likely that some of this "internally bound protein" resulted from DNA that landed next to free protein on the grid. Some internally bound protein was observed at a low frequency at each reaction time, although initiation of unwinding from internal regions of the DNA was never observed. We also examined blunt-ended duplex M13mp11 DNA (7244 bp) under identical conditions and found initiation of unwinding only from DNA ends (Fig. 2*C*).

At the ratio of helicase II to DNA used in these experiments, the majority (80 ± 5%) of the blunt-ended, linear pUC8 DNA was either completely unwound or fully native, with helicase II bound to the end. This suggests that initiation of unwinding is rate-limiting and that unwinding is rapid, once initiated, which is qualitatively consistent with our previous observations that unwinding, as detected by a gel retardation assay, appeared all-or-none (20). The earliest time that we detected completely unwound DNA molecules (see Fig. 1*F*) was 30 sec. Under the conditions that we used to fix and mount the DNA, the unwound ssDNA is coated with helicase II protein and shows some compaction relative to duplex DNA.

The possibility that a contaminating exonuclease produced a high-affinity ssDNA region for initiation is unlikely. We tested our helicase II preparations for single-stranded and duplex exonuclease activities, both 5′ and 3′, and found the levels to be below our detection limits (data not shown).

Initiation of Helicase II-Catalyzed Unwinding on Nicked Circular DNA. Runyon and Lohman (20) have shown that helicase II can unwind nicked circular pUC8 DNA (treated with DNase I), although the site of initiation was not determined. To examine whether unwinding is initiated at a nick, we used as the unwinding substrate, circular M13mp11 RF DNA, which contained a single unique nick introduced by the f1 gene II protein. In separate experiments, we have shown that singly nicked M13mp11 DNA molecules can be fully unwound by helicase II (data not shown). The singly nicked M13 DNA was treated with helicase II as above, the reaction was stopped after 30 sec, and the DNA was viewed by EM (see Fig. 2 *D–F*). Of 55 DNA molecules scored, 2 had no bound protein, 49 showed initiation of unwinding at only one site, and 4 showed initiation at two sites. Thirty-eight of the DNA molecules had been unwound by <20%, thus reducing the likelihood that in these molecules a single unwound region resulted from multiple initiation sites that had coalesced. The 4 molecules that showed two sites for unwinding may have resulted from molecules that had been multiply nicked during isolation. These observations, along with the fact that no unwinding was observed on supercoiled M13mp11 DNA (see below), suggest that the site for initiation of unwinding is the nick. This conclusion was further supported by a few molecules, such as those in Fig. 2 *E* and *F*, which clearly show two unwinding forks (arrows), in which two displaced single strands, presumably from the nicked + strand, as well as the single-stranded region on the continuous − strand are coated with helicase II protein. We also infer from these molecules that unwinding progressed bidirectionally from the single nick.

As a further test of whether unwinding could be initiated from internal regions of duplex DNA, supercoiled M13mp11 DNA (containing ≈7 ± 2% nicked circles) was treated with helicase II in unwinding buffer at the same ratio of 0.017 helicase II (monomer) per nucleotide for 30 sec and visualized by EM. Of 188 molecules analyzed, only 18 (9.6%) showed some unwinding, and all of these appeared relaxed, suggesting

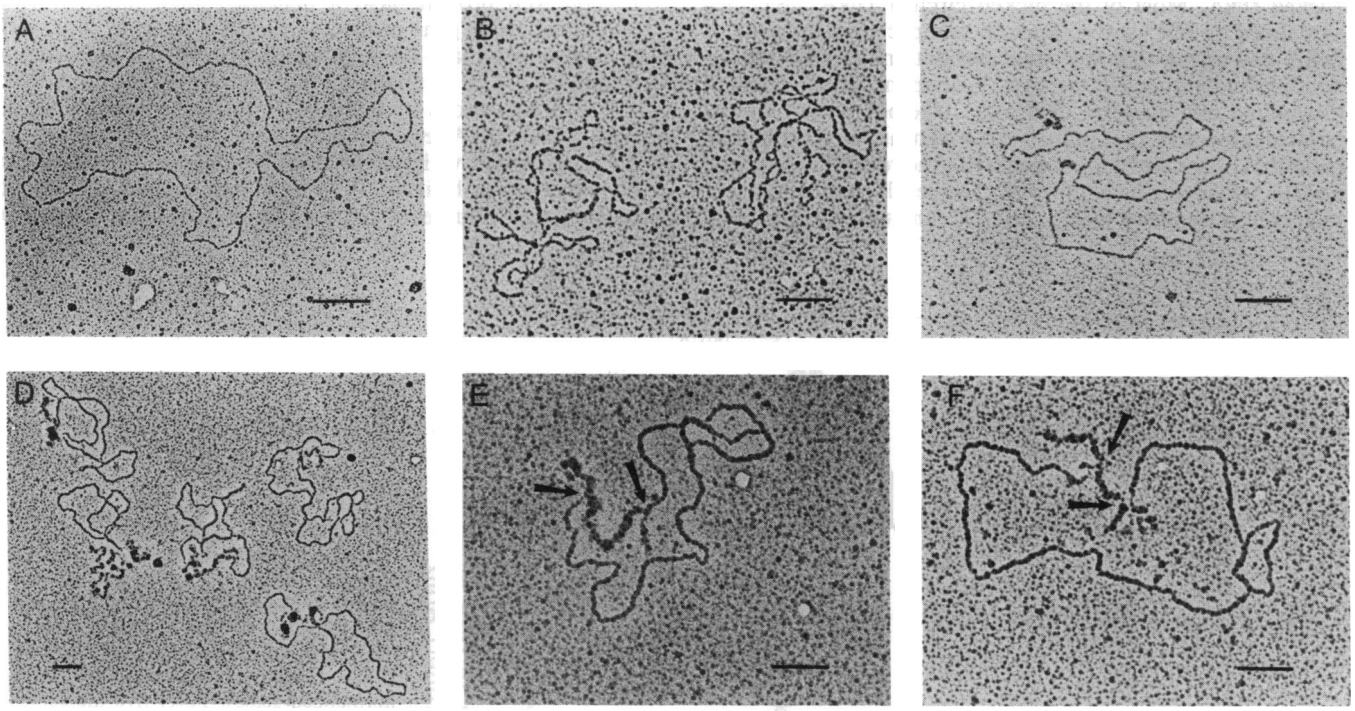

FIG. 2. Electron micrographs of M13mp11 DNA after incubation with helicase II for 30 sec as in Fig. 1. (*A*) Nicked circular DNA with no helicase II bound. (*B*) Supercoiled DNA. (*C*) Blunt-ended DNA. (*D–F*) Circular DNA nicked with f1 gene II protein. Arrows in *E* and *F* indicate the ssDNA–duplex DNA junctions resulting from initiation of unwinding at a nick. (Bars = 0.1 μm.)

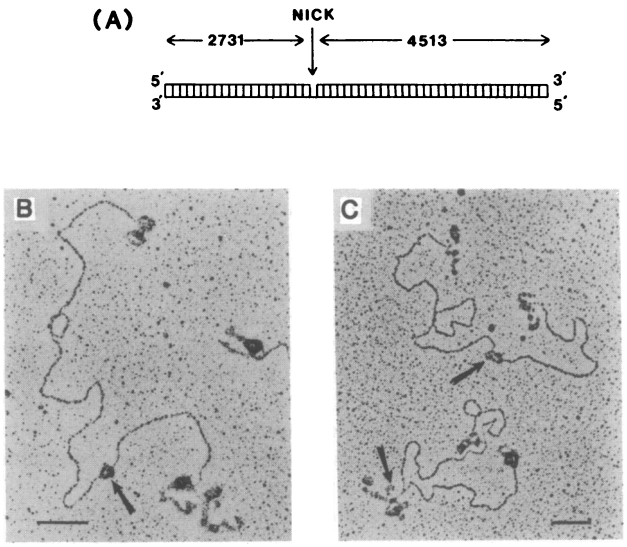

FIG. 3. (*A*) DNA substrate used to test whether helicase II can initiate unwinding of duplex DNA at a nick. The number of base pairs between the nick and each blunt end of the DNA is indicated. Electron micrographs of nicked linear M13mp11 DNA after initiation of unwinding by helicase II for 15 sec (*B*) and 30 sec (*C*). Reactions were performed as for Fig. 1. Arrows indicate sites of helicase II-initiated unwinding. (Bars = 0.1 μm.)

that they contained at least one nick, which may account for the fact that helicase II could initiate unwinding. Another 31 molecules showed some binding of helicase II protein but no unwinding. The percentage of molecules on which we observed unwinding (9.6%) is close to the percentage of relaxed molecules in the supercoiled population. These results indicate that helicase II has a clear preference for initiation of unwinding from blunt ends and nicks; however, we cannot rule out some small extent (<1–2%) of unwinding that may have initiated from internal duplex regions.

Mapping the Internal Sites of Initiation of Unwinding on Nicked DNA. Most of the nicked circular DNA molecules visualized by EM were sufficiently compacted at the point of DNA unwinding that we could not determine whether unwinding had initiated at the nick. To map the initiation site of unwinding and thereby determine whether or not it was coincident with the site of the nick, we linearized the uniquely nicked M13 circles by treatment with the restriction enzyme *Sna*BI, which leaves blunt ends. The resulting nicked linear molecule is depicted in Fig. 3*A*. The unique nick is located asymmetrically within the blunt-ended DNA molecule. The short and long duplex "arms" are 2731 and 4513 bp of 37% and 63% of the total length of the DNA molecule, respectively. Using this DNA, we mapped the site of initiation of unwinding, relative to the unique nick. These molecules were treated with helicase II as above, the reaction was stopped after 30 sec, and the molecules were visualized by EM (Fig. 3 *B* and *C*). On the majority of these molecules, unwinding had initiated at the two blunt ends as well as at a single internal site.

The location of the internal site of DNA unwinding was mapped by measuring the contour lengths of the DNA on each side of the internally unwound region. These measurements yield only an approximate location of the site of internal unwinding (within ±5% of the total DNA length) as discussed. Fig. 4 shows the locations of the sites of internal unwinding relative to the ends of the DNA molecule on a scale from 0–1, with the site of the unique nick located at 0.63. Greater than 80% of the sites of internal unwinding mapped to within 7.5% of the known site of the nick. The simplest interpretation of these results is that the nick is the site of initiation of internal DNA unwinding catalyzed by helicase II protein.

DISCUSSION

The EM study reported here clearly shows that *E. coli* helicase II protein can initiate unwinding of fully duplex DNA at nicks and blunt ends in a reaction requiring ATP and Mg^{2+} and that a preexisting region of flanking ssDNA is not required. Furthermore, initiation of DNA unwinding occurs extremely rarely, if at all, at internal duplex regions under the conditions used in our study, although we cannot rule out that helicase II might initiate unwinding from internal duplex DNA sites at higher protein concentrations. These results confirm and extend the studies of Runyon and Lohman (20), which demonstrated that helicase II can initiate unwinding on fully duplex and nicked circular DNA and unwind duplexes of at least 2700 bp in the absence of auxiliary proteins. The *E. coli* Rep protein, also a helicase, must interact with additional phage gene products (ϕX174 gene A or Ff gene II proteins) to initiate DNA unwinding from a nicked origin during ϕX174 or Ff phage rolling-circle replication (24, 25).

The observations of helicase II-catalyzed unwinding of fully duplex DNA reported by Runyon and Lohman (20) are not inconsistent with previous studies *in vitro* (7, 19), since higher ratios of helicase II to DNA are required to detect unwinding of blunt-ended or nicked DNA molecules *in vitro*

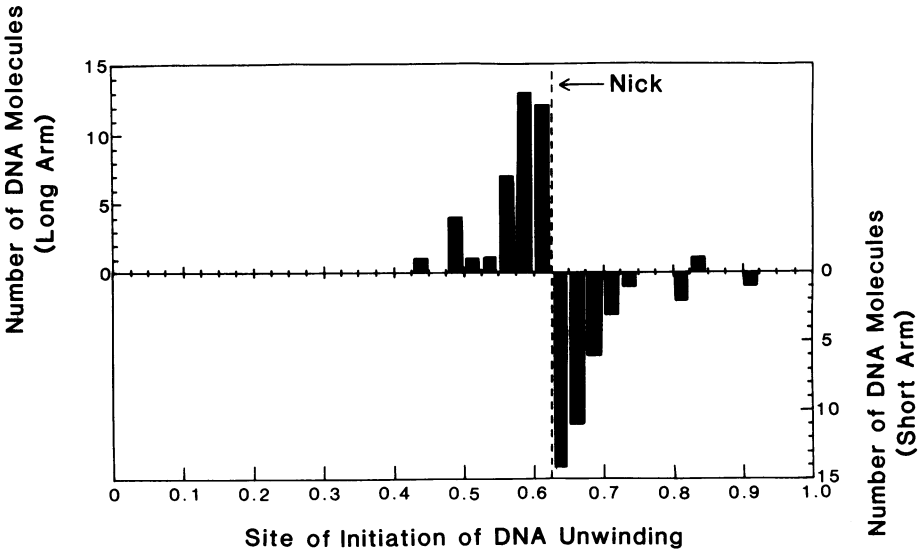

FIG. 4. Histogram of internal sites of helicase II-initiated unwinding on the nicked linear DNA substrate of Fig. 3*A* (DNA contour length normalized to 1). The site of the unique nick introduced by f1 gene II protein is indicated (0.63 on the normalized scale). Forty-five molecules were analyzed: 38 and 40 measurements were made from the short and long duplex arms, respectively; both arms were measured for 31 molecules.

(20). As discussed (20), high concentrations of helicase II protein (2–5 × 10^4 molecules per cell) are known to accumulate in *E. coli* after induction by DNA damage (3, 26–28), and those levels are comparable to the levels required in the experiments reported here and previously (20). Therefore, these observations are probably relevant under certain conditions *in vivo*. In this study, our goal was to examine DNA molecules on which unwinding had only initiated; we consequently used lower helicase II-to-DNA ratios (0.017 monomer per nucleotide) than is required to fully unwind all DNA in the population.

Previous studies have suggested that helicase II protein is required in direct proportion to the amount of duplex DNA unwound and, therefore, that the protein binds in stoichiometric amounts to the displaced ssDNA (19, 29). Our EM studies show that helicase II remains bound to the unwound ssDNA, thus preventing reannealing by serving as its own helix-destabilizing protein. In fact, the continuous binding of helicase II to the already strand-separated ssDNA may be necessary to sustain unwinding. From the fact that helicase II protein appears to saturate the ssDNA formed by the unwinding reaction, one might infer that helicase II binding to ssDNA is highly cooperative. However, this is not necessarily the case because helicase II possesses high affinity for ssDNA (30) and is in excess over the available ssDNA binding sites during the initial stages of unwinding. Our EM studies are consistent with the observation that binding of helicase II to duplex DNA is extremely weak (20, 30). Therefore, the high free concentration of helicase II in our studies insures that the ssDNA regions produced will be saturated with protein, even in the absence of cooperativity.

Current models for both methyl-directed mismatch and UvrABC excision repair in *E. coli* invoke helicase II as the agent that unwinds a region of DNA containing the damage site (12, 18, 31, 32). In methyl-directed mismatch repair, helicase II might initiate DNA unwinding either at a nick introduced in an earlier step or at the mismatch (18); however, before this report, there was no direct evidence that helicase II could initiate DNA unwinding at either of these sites. In studies of methyl-directed mismatch repair in *E. coli*, using a reconstituted system of purified proteins, Modrich and colleagues (13, 33) showed that a single nick in the DNA, which can be as much as 1000 bp from the site of mismatch, is essential for repair and that DNA excision requires helicase II protein. Runyon and Lohman (20) showed that helicase II can initiate on and fully unwind a nicked circular duplex DNA as long as 2700 bp, and in this report we have shown that helicase II can initiate unwinding at a nick. Therefore, our studies suggest that a nick in the DNA may provide the entry site for helicase II to initiate DNA unwinding in methyl-directed mismatch repair; however, this hypothesis remains to be tested.

Our studies may also bear on another aspect of the methyl-directed mismatch repair process—namely, the mechanism by which only the intervening DNA between the mismatch and the site of the nick is excised. The site of the mismatch is recognized by MutS protein (34), and the nick is introduced by MutH protein on the unmethylated strand at a GATC site (13, 35), with these two sites often separated by a few thousand bp (13, 33). The intervening DNA between these two sites is then thought to be unwound by helicase II and the gap filled in by DNA polymerase III (13). However, once the nick is introduced by MutH protein at the GATC site, the mechanism by which only the intervening DNA between the mismatch and the nick is unwound and removed is not known (36). Our studies suggest that helicase II-catalyzed unwinding of DNA progresses bidirectionally when initiated at a nick. Therefore, DNA unwinding by helicase II does not impose the directionality seen in the methyl-directed mismatch repair system. One possibility is that another protein becomes stably bound on one side of the nick, thus constraining helicase II to unwind unidirectionally from the nick. A likely candidate for this "clamp" is the MutH protein, which does remain bound to the GATC site after it introduces the specific nick on the unmethylated strand at this site (13). Of course, direct interactions between helicase II and other repair proteins may also be responsible for defining the directionality of helicase II-initiated unwinding from a nick.

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