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Unwinding of Nucleosomal DNA by a DNA Helicase*

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Angela K. Eggleston‡§, Timothy E. O'Neill¶‖, E. Morton Bradbury¶, and Stephen C. Kowalczykowski‡ ‡‡**

*From the ‡Sections of Microbiology and of Molecular and Cellular Biology and ¶Department of Biological Chemistry, University of California, Davis, California 95616 and the **Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545*

We have asked whether a DNA helicase can unwind DNA contained within both isolated native chromatin and reconstituted chromatin containing regularly spaced arrays of nucleosome cores on a linear tandem repeat sequence. We find that *Escherichia coli* recBCD enzyme is capable of unwinding these DNA substrates and displacing the nucleosomes, although both the rate and the processivity of enzymatic unwinding are inhibited (a maximum of 3- and >25-fold, respectively) as the nucleosome density on the template is increased. The observed rate of unwinding is not affected if the histone octamer is chemically cross-linked; thus, dissociation, or splitting, of the histone octamer is not required for unwinding to occur. The unwinding of native chromatin isolated from HeLa cell nuclei occurs both in the absence and in the presence of linker histone H1. These results suggest that as helicases unwind DNA, they facilitate nuclear processes by acting to clear DNA of histones or DNA-binding proteins in general.

In the eukaryotic nucleus, processes such as DNA replication, transcription, and recombination are highly regulated. One factor which affects these processes is the structure of the DNA, which is maintained in a highly condensed state by its assembly into chromatin. The mechanistic interplay between these nuclear processes and chromatin structure is complex and has proven difficult to elucidate. Recent investigations using defined chromatin templates have demonstrated that chromatin structure plays essential roles in the mechanism of transcription activation (reviewed in Refs. 1, 2). The effect of chromatin structure on DNA replication and recombination has not been extensively studied *in vitro*, although genetic studies in yeast have indicated that alteration of chromatin structure influences the recombinogenicity of DNA sites (see, for example, Refs. 3–6).

Direct measurement of the effect of nucleosomes on DNA replication was conducted *in vitro* by Bonne-Andrea *et al.* (7). Using the entire complement of purified T4 bacteriophage replication proteins, the replication of plasmid DNA-containing nucleosomes was examined. This hybrid system was used because such a defined, well-characterized system offered a means by which modest changes in activity could be detected. On a DNA template which was moderately reconstituted with nucleosomes (~3/4.7 kb[1] molecule), the replication fork was able to pass through the nucleosome structure after a certain amount of pausing, resulting in an overall slower rate of synthesis compared to that on protein-free DNA. Interestingly, this reaction absolutely required a DNA helicase, the T4 dda protein, presumably to assist in the displacement of protein blockades on the duplex DNA ahead of the replication fork. The data were interpreted to show that the core particle remained intact throughout the replication process and that the histone octamer interacted, at least transiently, with ssDNA. Other studies have indicated that histone octamers do not bind ssDNA (8), although the possibility that the histones formed a suboctameric association with ssDNA was not excluded. Alternatively, these observations may be explained by either binding of the histone octamer to secondary structure within the replicating DNA, intramolecular transfer of the octamer (9, 10), or transient displacement of the octamer followed by diffusion back onto the DNA (11).

To address how the packaging of DNA into chromatin affects recombination, DNA strand exchange promoted by the *Escherichia coli* recA protein was examined *in vitro* using substrates which were reconstituted with either *E. coli* HU protein or rat liver core histones (12, 13). The presence of HU protein on the dsDNA recipient molecule did not affect the overall ability of recA protein to form paranemic (non-intertwined) joint molecules, but it did inhibit the formation of topologically linked plectonemic molecules. If HU protein was bound to the ssDNA donor molecule to which recA protein binds initially, neither paranemic nor plectonemic joint molecule formation was affected. On templates containing eukaryotic chromatin structure, homologous pairing occurred on templates reconstituted at histone/DNA weight ratios up to 1.6:1 (nearly twice the *in vivo* ratio), although DNA strand exchange was prevented. The addition of histone H1 to the chromatin DNA inhibited even the initial homologous pairing of the DNA molecules. These results suggest that another factor may be required *in vivo* to disrupt this type of nucleoprotein structure so that the exchange of homologous DNA strands can occur.

One candidate for such a factor is a DNA helicase. Both prokaryotic and eukaryotic cells contain many DNA helicases, which possess different substrate specificities and which function in various biological processes. In principle, any DNA helicase must contend with proteins which organize the chromosome as well as with other DNA-binding proteins. We have utilized a heterologous system assembled from purified components to characterize how DNA helicase activity is

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§ Present address: Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Potters Bar, Hertfordshire EN6 3LD, United Kingdom.

‖ Present address: Dept. of Food Science and Technology, University of California, Davis, CA 95616.

‡‡ To whom correspondence should be addressed. Tel.: 916-752-5938; Fax: 916-752-5939; E-mail: sckowalczykowski@ucdavis.edu.

[1] The abbreviations used are: kb, kilobase(s); ss, single-stranded; ds, double-stranded; bp, base pair(s); PMSF, phenylmethylsulfonyl fluoride; SSB, single-stranded DNA binding.

affected by the presence of nucleosomes.

In *E. coli*, the primary helicase involved in homologous recombination is recBCD enzyme, a heterotrimeric, 330-kDa protein (for reviews, see Refs. 14–16). We chose to use this enzyme for these studies because its helicase activity is well defined with regard to reaction requirements and enzymatic parameters. Although initially identified as a potent, ATP-dependent nuclease capable of degrading DNA exo- and endonucleolytically (17–19), recBCD enzyme is also a highly processive DNA helicase (20, 21) capable of unwinding an average of 30 kb/binding event *in vitro* (22). Unwinding occurs at a rate of approximately 1000–1500 bp/s at 37 °C (23, 24) and requires the hydrolysis of 2–3 ATP molecules/bp unwound (25). Unlike most helicases involved in replication or repair, recBCD enzyme catalyzes DNA unwinding without the requirement for an accessory factor or a specialized substrate, such as a tailed molecule having a defined polarity. Thus, this enzyme is an ideal candidate for the study of how helicase activity is influenced by the presence of chromatin structure.

We assessed the DNA unwinding activity of recBCD enzyme on two types of linear chromatin template. The first was a completely defined array of nucleosome cores assembled onto a template containing tandem repeats of the 207-bp nucleosome positioning sequence derived from the 5 S rRNA gene of *Lytechinus variegatus* (26). The reconstitution of regularly spaced arrays of nucleosome cores onto this sequence from donor histone octamers has been thoroughly characterized (26–29). The second chromatin template was isolated from HeLa cell nuclei. The ability of recBCD enzyme to unwind both of these substrates was primarily monitored using a fluorometric assay, and the results were confirmed by analyzing the products of an unwinding reaction on neutral sucrose gradients. The experiments described below demonstrate that recBCD enzyme can generate ssDNA products from both types of chromatin template via displacement of histone proteins from the DNA substrate. They also suggest a general role of helicases in displacing DNA-binding proteins.

EXPERIMENTAL PROCEDURES

Nucleic Acids—The linear DNA substrate used in reconstitution studies was derived from plasmid p5S207–18 (26), which consists of 18 repeats of the 207-bp nucleosome positioning sequence of the *L. variegatus* 5 S rDNA sequence cloned into the pAT153 vector. The specific tandem repeat DNA (~3800 bp) was obtained by *Hha*I digestion of p5S207–18 and isolation of desired fragment by fast protein liquid chromatography on Mono-Q HR5/5 (Pharmacia Biotech Inc.). The linear DNA fragment was quantitated using an A_{260} of 20 = 1 mg/ml. pBR322 DNA was prepared as described (20), and the molar nucleotide concentration was quantitated using an extinction coefficient at 260 nm of 6500 M^{-1} cm^{-1}. The molar concentration of DNA ends was determined by dividing the molar nucleotide concentration by 3800 (207_{18} HhaI fragment), 4361 (pBR322), or 4000 (native chromatin) bp/molecule.

Proteins—RecBCD enzyme was purified as described (23, 31). The concentration of protein was determined using an extinction coefficient of 4×10^5 M^{-1} cm^{-1} at 280 nm (23). This protein preparation has specific activities of 4.2×10^4 helicase units/mg (as defined in Ref. 23) and 3.1×10^5 nuclease units/mg (as defined in Ref. 32); its apparent binding stoichiometry (used to calculate the amount of functional enzyme) is 3 recBCD enzyme molecules/DNA end (see Ref. 23). SSB protein was purified from strain RLM727 as described (33) and was quantitated using an extinction coefficient of 3×10^4 M^{-1} cm^{-1} at 280 nm (34).

Histone octamers were prepared from HeLa cell nuclei as described (35). Cross-linking of the histone octamers with dimethyl suberimidate was performed as described (36).

Restriction enzymes and DNA modification enzymes were obtained from Pharmacia, New England Biolabs, and Boehringer Mannheim.

Reconstitution of the Nucleosomal Substrates—Nucleosome cores were reconstituted onto linearized 207_{18} DNA by salt step dialysis (29). Histone octamers and DNA substrate were mixed at varying ratios (final A_{260} of 5) in 2M NaCl, 10 mM Tris-HCl, pH 7.4, and 0.2 mM EDTA at 4 °C. Small volumes were dialyzed against decreasing NaCl concentration (2 M, 1.5 M, 0.75 M, 0.5 M NaCl in 10 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, and 0.1% Nonidet P-40) in 1–2 h steps, and a final overnight step to 10 mM NaCl was performed in the same buffer without Nonidet P-40. Extensively cross-linked histone octamers were reconstituted onto linearized 207_{18} DNA using the same method, except that salt step dialysis was begun at 1 M NaCl instead of 2 M NaCl. The integrity of the reconstituted chromatin was confirmed by digestion with micrococcal nuclease (data not shown; (35)). The nucleosomal templates are designated as C_n, where n indicates the weight ratio (histone/DNA) at which the reconstitution was done (*e.g.* $C_{0.4}$ = a 0.4:1 histone/DNA weight ratio during the reconstitution procedure).

Isolation of Native Chromatin—HeLa cell nuclei were digested with micrococcal nuclease (4 units/mg; Worthington) at a DNA concentration of 2 mg/ml for 12 min at 37 °C in 10 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$, and 0.25 mM PMSF. Nuclei were lysed by overnight dialysis against 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 0.25 mM PMSF. The soluble chromatin was fractionated on 12 ml 5–25% sucrose gradients in a SW40 rotor at 26,000 revolutions/min for 16 h at 4 °C. Fractions in which the DNA length averaged approximately 4 kb were pooled and dialyzed against 10 mM Tris-HCl, pH 7.4, 0.2 mM EDTA, and 0.1 mM PMSF.

Fluorometric Helicase Assay—The unwinding of duplex DNA by recBCD enzyme was monitored using a fluorescent assay (23). The standard reaction conditions consisted of 20 mM Tris acetate, pH 7.5, 1 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 20 mM NaCl. Unless otherwise noted, the DNA concentration was nominally 10 μM nucleotide (see below), and SSB protein was added to 2 μM (a value 3-fold in excess to the amount required to bind the ssDNA present if the dsDNA is fully unwound). RecBCD enzyme was then added to the indicated concentration. Under these conditions (*i.e.* in the absence of ATP), the enzyme binds the ends of the duplex DNA, although it is unable to unwind it (37, 38). After equilibrating the reaction to 25 °C, unwinding was synchronously initiated by adding ATP to 3 mM final concentration. At this ratio of ATP to Mg^{2+} concentration, the dsDNA exonuclease activity of recBCD enzyme is largely suppressed (31). The reaction was conducted in a total volume of 350 μl, and fluorescence measurements were collected using a Shimadzu RF5000U spectrofluorophotometer. The initial rate of unwinding was calculated as described (23).

Since reconstitution of the nucleosomal templates involved dialysis, the concentration of the reconstituted DNAs was not known precisely. Any resultant variation, however, is accounted for by the following control reaction. To obtain a value for the total amount of fluorescence quenching possible, an equivalent nominal concentration of the protein-free or chromatin DNA was heat-denatured at 95 °C for 7 min and was immediately quenched in ice water. This DNA was then added to a cuvette containing all of the remaining components of the helicase assay except recBCD enzyme, and the fluorescence change after the addition of the heat-denatured DNA was measured. This value indicates directly the maximal amount of fluorescence quenching expected if the DNA is fully unwound and was independently determined for each DNA sample.

Sucrose Gradient Fractionation and Quantitation—Reactions (520 μl) were performed in buffer containing 25 mM HEPES, pH 7.5, 1 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 100 mM NaCl. The concentrations of DNA (nucleotide), SSB protein, and recBCD enzyme were 60 μM, 8 μM, and 16.1 nM, respectively. (As a control, recBCD enzyme was omitted from replicate reactions.) After adding ATP to a final concentration of 3 mM, the reaction was incubated at room temperature (~26 °C) for 5 min (protein-free DNA) or 30 min (H1-containing chromatin). Reactions were stopped by adding EDTA to a final concentration of 5 mM, followed by storage on ice. 500 μl of each reaction was loaded onto prechilled 12-ml gradients containing 5–30% sucrose in 10 mM HEPES, pH 7.5, 5 mM EDTA, and 0.25 mM PMSF. The gradients were centrifuged in a SW40 rotor at 26,000 revolutions/min at 4 °C for 16 h.

At the end of the run, the gradients were fractionated into ~0.5 ml aliquots. To portions of each, a 0.1 volume of 2% SDS, 50 mM EDTA was added. The sample was then extracted once with phenol/chloroform and ethanol-precipitated. The pellet was resuspended in 0.3 M NaOH and heated at 65 °C for 1 h. 20 × SSC (30) was added to a final concentration of 6×, and the samples were applied to a nylon membrane. The membrane was probed with 5′ end-labeled DNA made from the same native chromatin preparation which had been extracted with phenol/chloroform. Radioactivity was quantitated using a Fuji BA1000 PhosphorImaging system. Some signal loss was observed when chromatin DNA was unwound by recBCD enzyme. We are not certain as to the cause of this, but it is likely that since the enzyme's translocation rate is reduced in the presence of bound histone octamers, chromatin DNA is nicked

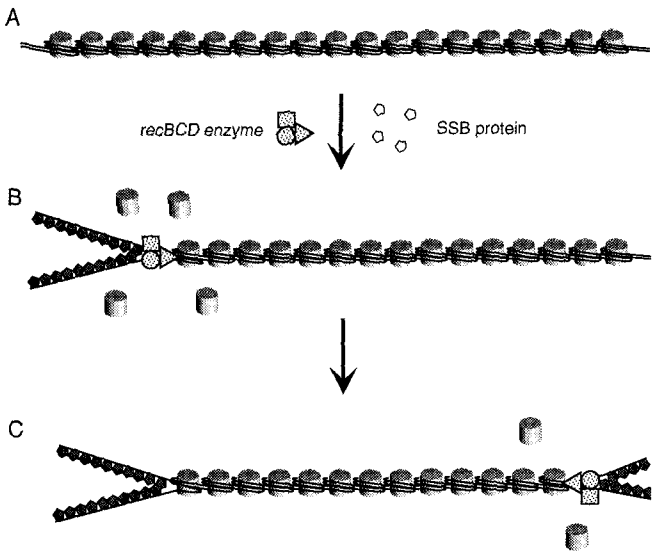

FIG. 1. **Model for unwinding of nucleosomal templates by recBCD enzyme.** Initially, the linear duplex DNA template is either protein-free (not shown) or contains bound histone octamers (*cylinders*, *A*). A fully reconstituted template is shown, but the density of nucleosomes is manipulated experimentally. RecBCD enzyme (*square/circle/triangle*) binds to the end of a DNA molecule and initiates unwinding when ATP is added. This unwinding disrupts the association of the histone octamers and DNA and facilitates the binding of SSB protein (*pentagons*) to the newly formed ssDNA (*B*). Although unwinding cannot reinitiate from an end which has been unwound by greater than 25 nucleotides, both ends of the molecule can be utilized, as illustrated by the binding of a second enzyme molecule to the end which was not unwound initially (*C*). This process is detected using a fluorometric helicase assay, which measures the quenching of the intrinsic fluorescence of SSB protein when it binds to ssDNA (indicated by the change in shading of the pentagons).

more frequently (31), and these smaller ssDNA fragments may be less efficiently precipitated during the sample preparation.

RESULTS

RecBCD Enzyme Unwinds Duplex DNA Reconstituted with Nucleosomes—When SSB protein binds to ssDNA, its intrinsic tryptophan fluorescence is quenched. This property was exploited to measure, in a continuous kinetic assay, the unwinding of dsDNA by recBCD enzyme (23). The fluorometric helicase assay was adapted for use with chromatin DNA; a diagram of this process as it is proposed to occur on a nucleosomal template is shown in Fig. 1. The helicase is incubated with linear chromatin DNA in the presence of excess SSB protein. As the DNA is unwound, SSB protein rapidly binds the ssDNA tails, resulting in a fluorescence decrease which is directly proportional to the amount of DNA unwound.

Fig. 2*A* shows raw fluorescence data obtained using a subsaturating concentration of recBCD enzyme and *in vitro* reconstituted chromatin templates. In these experiments, the histone/DNA weight ratio (n) during the reconstitution procedure ranged from 0 (protein-free DNA) to 1 (highly reconstituted, with essentially every positioning sequence on all DNA molecules occupied). We interpret the quenching of SSB protein fluorescence as direct evidence that the histones are being displaced from the DNA as it is unwound, although other models are possible (see "Discussion"). From such data, the apparent rates of unwinding were calculated as described under "Experimental Procedures" and are plotted in Fig. 2*B*. As the nucleosome density on the template increases, the rate of unwinding decreases (●). The inhibitory effect of the presence of nucleosomes at the highest levels of reconstitution lowers the rate of unwinding to ~30% of that obtained with protein-free DNA (10 ± 2.6 *versus* 33 ± 6.6 nm bp/s). The maximum inhibition of unwinding occurs at physiologically relevant nucleosome densities (n ~0.8–1.0). We conclude that since even highly reconstituted DNA is acted upon (Fig. 2 and data not shown), chromatin DNA can be unwound and nucleosomes are displaced by the helicase activity of recBCD enzyme.

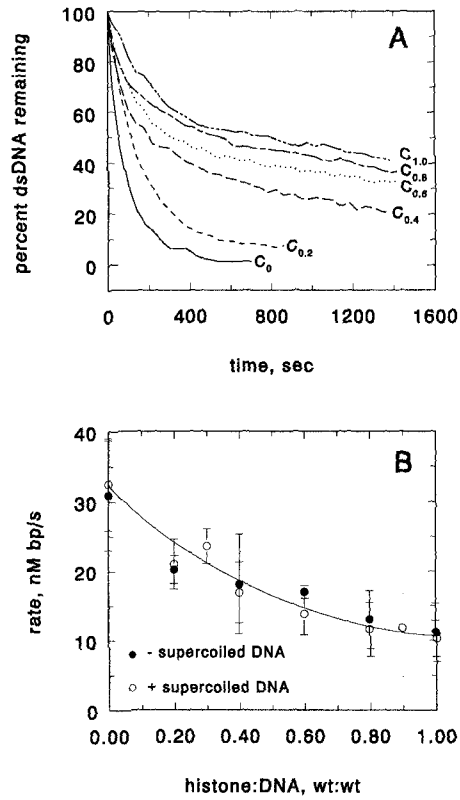

FIG. 2. **Unwinding of reconstituted templates as a function of nucleosome density.** The reaction contained 20 mM Tris acetate, pH 7.5, 1 mM $Mg(OAc)_2$, 0.1 mM dithiothreitol, 20 mM NaCl, 2 μM SSB protein, 10 μM nucleotide (2.6 nM DNA ends) linear 207_{18} DNA substrate, and 0.5 nM (0.17 nM functional) recBCD enzyme. This concentration of enzyme corresponds to <0.1 functional enzyme molecule/DNA end. After equilibrating the reaction to 25 °C, ATP was added to a final concentration of 3 mM to allow recBCD enzyme to initiate unwinding of the DNA. When indicated, 10 μM nucleotide supercoiled pBR322 DNA was also included. *A*, shows the raw data obtained using the fluorometric helicase assay, with the following C_n values for the reconstituted substrate: 0, *filled circles*; 0.2, *open circles*; 0.4, *filled triangles*; 0.6, *open triangles*; 0.8, *filled diamonds*; 1.0, *open diamonds*. In *B*, the initial rates of unwinding were calculated as described under "Experimental Procedures." Reactions without supercoiled DNA (●); reactions with supercoiled DNA (○).

One trivial explanation for the reduction in helicase activity might be that the displaced histone octamers were directly affecting the enzyme by binding to it. To test this hypothesis, reactions were also performed in the presence of an equimolar amount of supercoiled pBR322 DNA (Fig. 2*B*, ○). This DNA can act as a trap to bind displaced histones but will not interfere with the unwinding reaction because supercoiled DNA, lacking an end, is not detectably bound by recBCD enzyme (23, 39). As expected, the addition of this DNA does not affect the rate of unwinding on protein-free DNA (33 ± 6.6 *versus* 31 ± 7.9 nM bp/s), nor is there an effect even with highly reconstituted chromatin substrates (Fig. 2*B*). In addition, supplementation of reactions containing either protein-free DNA or moderately reconstituted DNA ($C_{0.2}$) with purified histone octamers does not affect the rate of unwinding (26 *versus* 24 and 20 *versus* 18 nM bp/s, respectively; data not shown). Alternatively, the displaced histones may interfere with the binding of SSB protein to ssDNA. Direct titrations of SSB protein and free histone octamers with ssDNA were performed to determine whether

histones compete with SSB protein for ssDNA-binding sites. When SSB protein is bound to ssDNA and histones are subsequently added, a gradual decrease in the amount of fluorescence quenching is observed with increasing molar concentrations of free histone octamers; however, even at a 0.7 molar ratio (histone/DNA), 85% of the expected fluorescence decrease is obtained (data not shown). Similarly, the addition of both proteins simultaneously results in the maximal fluorescence quenching expected (data not shown). Thus, displaced histones do not inhibit unwinding to a significant extent by either binding to recBCD enzyme, preventing the binding of SSB protein, or renaturing the unwound ssDNA.

To confirm that the enzyme was acting catalytically on these substrates, the reactions were repeated using a 4-fold higher, but still subsaturating, concentration of enzyme (2 nM total enzyme; 0.25 functional enzyme molecule/DNA end). As expected, this increase in enzyme concentration results in a proportionate increase (4-fold) in the apparent rate of the reaction at all reconstitution ratios (data not shown).

Nucleosomes Reduce the Processivity of RecBCD Enzyme Helicase Activity—RecBCD enzyme initiates unwinding only on duplex molecules which have ssDNA tails of ≤25 nucleotides (37). Because reinitiation cannot occur on substrates which have been unwound greater than 25 nucleotides, the properties associated with a single cycle of enzyme association, translocation, and dissociation are easily determined (22). Since the fluorometric helicase assay measures an average property of all the DNA molecules present in the reaction (the average number of base pairs unwound per DNA molecule), the extent of the reaction, if less than 100%, is twice (because there are two binding sites for the enzyme per DNA molecule) the average processive distance traveled by the enzyme per binding event. The processivity of recBCD enzyme helicase activity under a variety of experimental conditions is known. Under conditions similar to those used here, the enzyme unwinds an average of 30 kb/end before dissociating (22).

To obtain the unwinding processivity, the percentage of the total DNA unwound during the reaction (*i.e.* the extent of unwinding) was calculated (Fig. 3*A*). All of the 207_{18} DNA substrate (~3.8 kb in length) should be readily unwound unless the processivity of the enzyme is reduced >15-fold (30 kb/end/1.9 kb/end) by the presence of nucleosomes. This expectation is true for reconstitution ratios of less than $C_{0.4}$ but not for higher reconstitution ratios (Fig. 3*A*). Thus, the average distance that recBCD enzyme can unwind is reduced by the presence of nucleosomes, with higher degrees of reconstitution demonstrating a greater effect. Even near saturating nucleosome density, however, a majority (60%) of the total DNA is unwound.

To illustrate the dramatic effect nucleosome structure has on processivity, the values for the extent of unwinding were converted to values of N (the average number of base pairs unwound per DNA end; Fig. 3*B*). Because extent values near 100% are uninformative for processivity determinations, we cannot estimate the processivity at low reconstitution ratios ($<C_{0.4}$). At the highest reconstitution ratios, when approximately 60% of the DNA is unwound, N is ~1.2 kb/end, which corresponds to a 25-fold decrease in processivity compared to the value obtained on protein-free DNA (22). Additionally, the use of a 4-fold higher enzyme concentration (which is still subsaturating with respect to the concentration of DNA ends in the reaction) yields results for the processivity parameter N which are within the experimental error of these values (data not shown).

Because the fluorescent helicase assay measures the average properties of a DNA population, it cannot distinguish between

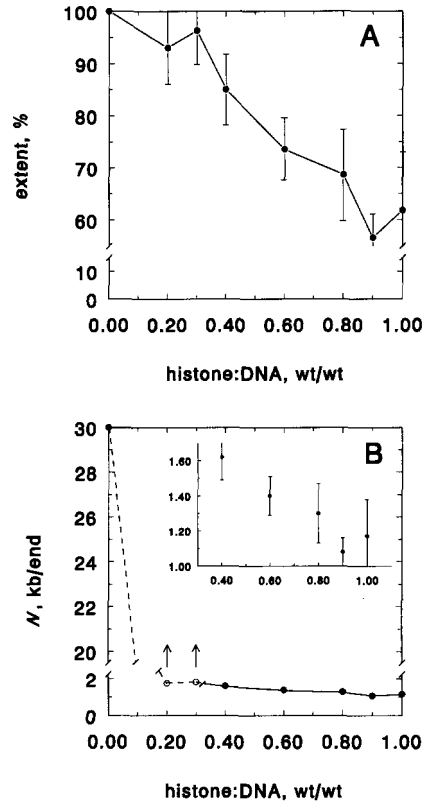

FIG. 3. **Processivity of unwinding on reconstituted DNA templates.** In *A*, the extents of the unwinding reactions (*i.e.* the percentage of DNA unwound) shown in Fig. 2 are plotted. The values of N, the average distance unwound by the helicase per binding event, were calculated as described previously (22) and are plotted in *B*. For extents which are approximately 100% (○), it is impossible to calculate an accurate processivity value (indicated by the *arrows*). At the higher reconstitution ratios, however, the extents of unwinding are consistently less than 100% (●); these data provide quantitative information regarding the processivity of the enzyme under these conditions.

differences in the ability of subpopulations of molecules to be unwound. The susceptibility of the template molecules to unwinding was examined on an agarose gel. If a population of molecules is resistant to recBCD enzyme helicase activity, it will migrate in the position of the starting material; otherwise, all of the substrate band will disappear as the molecules are unwound. Consistent with the latter proposal, the protein-free DNA substrate is rapidly converted into a heterogeneous smear of unwound ssDNA fragments (data not shown; see Ref. 31, for an example using M13 DNA). The disappearance of the highly reconstituted ($C_{0.8}$) DNA is less rapid, but 90 ± 10% of the substrate DNA disappears after 10 min of incubation. Since a subsaturating concentration of recBCD enzyme (0.1 functional enzyme molecule/DNA end) was used, this result confirms that the enzyme is acting catalytically. If a higher concentration of enzyme is used, all of the DNA substrate is also unwound (data not shown).

Nucleosomal DNA Sequesters RecBCD Enzyme—One explanation for the decreased rate of unwinding on nucleosomal DNA is that the rate of unwinding within the linker DNA is similar to that of the protein-free DNA, but that the nucleosomes slow or impede the progress of the enzyme (*i.e.* the structures cause the enzyme to pause) (see "Discussion"). Thus, the more blockades, in the form of histone octamers, which the enzyme encounters, the slower the apparent rate. In effect, the presence of nucleosomes sequesters an enzyme molecule on the DNA for a period of time.

To determine whether the enzyme was being sequestered, an

TABLE I
Effect of exposure of recBCD enzyme to reconstituted chromatin DNA on subsequent unwinding

Unwinding was measured as described in the legend to Fig. 2, except that a second aliquot of DNA was added after completion of unwinding of the first aliquot. The extent (*i.e.* the percentage of DNA unwound) and the initial rate of unwinding for each aliquot of DNA are shown.

Order of addition		First aliquot		Second aliquot	
First	Second	Extent	Rate	Extent	Rate
		%	*nM bp/s*	%	*nM bp/s*
C_0	C_0	96 ± 6	21.1 ± 0.5	100 ± 3	18.3 ± 2.8
C_0	$C_{0.8}$	96 ± 6	26.5 ± 8.0	50 ± 11	6.0 ± 2.3
$C_{0.8}$	$C_{0.8}$	70 ± 6	8.0 ± 0.9	25 ± 2[a]	1.2 ± 0.2
$C_{0.8}$	C_0	64 ± 10	6.8 ± 0.7	100 ± 3	4.8 ± 1.3

[a] Due to the slow rate of unwinding, this result is probably an underestimate since a level plateau value is not achieved.

TABLE II
Unwinding of native chromatin DNA by recBCD enzyme

Unwinding was measured as described in the legend to Fig. 2, except that the substrate was native chromatin isolated from HeLa cell nuclei, and the salt concentration was varied as indicated.

Substrate	[Nacl]	Observed extent	Corrected extent	Rate
	mM	%		*nM bp/s*
Protein-free DNA	0	75 ± 3	100	10.8 ± 0.9
	100	67 ± 1	100	18.7 ± 0.8
	200	54 ± 1	100	5.6 ± 0.1
H1-depleted chromatin	0	42 ± 6	56	5.9 ± 0.3
	100	44 ± 8	66	8.5 ± 1.7
	200	30 ± 1	56	2.6 ± 0.4
H1-containing chromatin	0	22 ± 1	29	1.1 ± 0.1
	100	38 ± 9	57	4.7 ± 0.2
	200	28 ± 4	52	1.7 ± 0.8

order of addition experiment was performed. DNA, either protein-free (C_0) or highly reconstituted ($C_{0.8}$), was unwound using a substoichiometric amount of enzyme (<0.1 functional enzyme molecule/DNA end). After the reaction was complete, a second aliquot of DNA, either C_0 or $C_{0.8}$, was added. The extent and rate data are summarized in Table I. When the DNA in both aliquots is free of nucleosomes, all of the DNA is unwound (*i.e.* the extent is ~100%), and the rate of unwinding of the second aliquot is slightly slower than that of the first aliquot due to the time-dependent loss of helicase activity when recBCD enzyme is incubated in dilute solution (23). If C_0 DNA is unwound to completion and then $C_{0.8}$ DNA is added, both DNAs are unwound to the extent and at the rate observed when either DNA is unwound alone. These results show that the activity of the enzyme is not significantly affected as a result of repeated cycles of unwinding.

The situation is strikingly different when a reconstituted chromatin template ($C_{0.8}$) is unwound initially, however. If the second aliquot of DNA is also $C_{0.8}$, the rate of unwinding of the second aliquot of DNA is reduced ~5-fold, and the extent of unwinding is apparently reduced by ~60% (although this reduction may be an overestimate because this reaction does not reach a distinct end point). A more dramatic effect is observed when the second aliquot contains protein-free DNA. If all of the recBCD enzyme molecules have completed unwinding of the first DNA substrate, have been unaffected in the process, and are free in solution, then the aliquot of C_0 DNA should be unwound at the rate and to the extent expected if the enzyme had not been previously exposed to the reconstituted DNA. Instead, the rate of unwinding is reduced 5-fold, although the reaction still goes to completion. This result indicates that the concentration of enzyme available to unwind this DNA is less than that present at the start of the reaction, suggesting that most (~80%) of the enzyme molecules have been sequestered onto the DNA containing nucleosomes or have been otherwise affected with regard to their ability to initiate unwinding on subsequent molecules (see "Discussion"). The enzyme molecules which are free in solution at the time the second DNA is added are able to act catalytically on that DNA; hence, all of the C_0 DNA is unwound.

RecBCD Enzyme Helicase Activity on Nucleosomal DNA Does Not Require Dissociation of the Histone Octamer—Several models for the transcription of nucleosomal DNA templates by RNA polymerase postulate that the histone octamer dissociates, or splits, in some fashion to allow the enzyme to progress along the DNA (for an overview, see Refs. 2, 41). Recent experiments, however, indicate that T7 RNA polymerase can transcribe through continuous arrays of nucleosome cores reconstituted from cross-linked histone octamers (36). Therefore, histone octamer dissociation is not required for transcription through nucleosome cores.

To examine the role of histone octamer dissociation on the helicase activity of recBCD enzyme, nucleosome cores were reconstituted onto linear 207_{18} DNA at a ratio of 0.8:1 using donor histone octamers which had been extensively cross-linked with dimethyl suberimidate (36). If octamer dissociation is required for recBCD enzyme to gain access to and separate the DNA strands, unwinding would be inhibited. Using 2 nM recBCD enzyme, the initial rate of unwinding of the chromatin substrate containing the chemically cross-linked histone octamers is not significantly different than that which is observed with the non-cross-linked substrate (40 ± 5 *versus* 49 ± 6 nM bp/s, respectively; data not shown). The extent of unwinding is also unaffected (49 ± 2 *versus* 46 ± 2%, respectively; data not shown). This result demonstrates that dissociation of the histone octamer is not necessary for helicase activity on nucleosomal templates.

Isolated Native Chromatin Is Unwound by RecBCD Enzyme Helicase Activity—To demonstrate that the helicase could unwind a physiological substrate, native chromatin ~4 kb in length (*i.e.* similar to the length of the 207_{18} template used to reconstitute chromatin DNA) was isolated from HeLa cell nuclei. This chromatin DNA was divided in three fractions. One fraction ("H1-containing chromatin") was untreated. A second fraction ("H1-depleted chromatin") was stripped of linker histone H1 by incubation with salt. Finally, protein-free DNA to serve as an internal control was generated by extensive phenol extraction of H1-depleted chromatin.

Using the fluorometric assay, we found that recBCD enzyme was able to unwind the native chromatin substrate (Table II). The rate of unwinding of the phenol-extracted native DNA is lower than that observed with the protein-free 207_{18} template. This result suggests that the DNA pool is intrinsically less suitable for unwinding by recBCD enzyme, perhaps due to fraying or degradation of the DNA ends or to internal nicking by micrococcal nuclease during the preparation and isolation of this DNA. The rate of unwinding of the H1-depleted native chromatin DNA at 0 mM NaCl is also less than that obtained with the reconstituted chromatin (at 20 mM NaCl); nevertheless, this chromatin, which should be roughly equivalent in nucleosome density to the highly reconstituted templates used previously, is unwound at a rate which is 50% that of the protein-free DNA (5.9 ± 0.3 *versus* 10.8 ± 0.9 nM bp/s, respectively), demonstrating good agreement between the two types of substrates. In addition, the extent of unwinding of the H1-depleted chromatin is similar to the previous results using reconstituted substrates (Table II). For the native chromatin preparation, the protein-free DNA is unwound to an extent of only 75%, rather than 100%. Assuming that this value represents the maximum amount of unwinding achievable with this

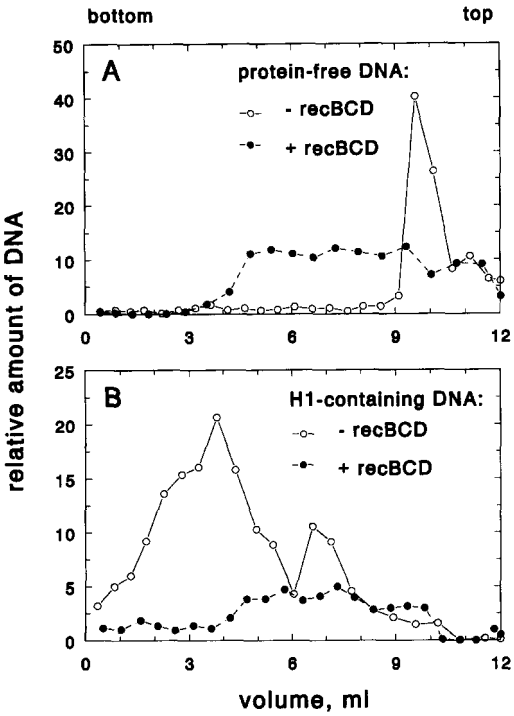

FIG. 4. **Characterization of the DNA products of a native chromatin unwinding reaction by sucrose gradient fractionation.** An unwinding reaction containing protein-free DNA (*A*) or H1-containing chromatin (*B*) was conducted as described under "Experimental Procedures." At a time sufficient for complete unwinding, the reaction was stopped, and a portion of each reaction was centrifuged through neutral sucrose gradients. The gradients were fractionated and the DNA content was identified as described under "Experimental Procedures." RecBCD enzyme omitted (○); recBCD enzyme included (●]).

DNA, the observed extent of 42% for the H1-depleted chromatin corresponds to ~60% of the maximum (the "corrected" extent), in agreement with the highly reconstituted chromatin substrates. One unexpected finding was that H1-containing chromatin could be unwound, albeit at a lesser rate than H1-depleted chromatin (Table II).

These reactions were conducted at varying NaCl concentrations to assess the effect of chromatin condensation on the rate of unwinding. At low salt concentration, the DNA will be at its most extended state, whereas increasing salt concentrations will cause the nucleosomes to fold into higher order structures. When the salt concentration was increased to 100 mM, the rate of unwinding of the chromatin DNAs increased (Table II). This result is not entirely unexpected since the rate of recBCD enzyme helicase activity on protein-free DNA increases 2-fold from 4 to 80–100 mM NaCl (Table II (23, 24)). At even higher salt concentrations (200 mM NaCl), the rate of unwinding decreases to a value ~30% of that at 100 mM NaCl. Because the effect of salt in these reactions appears to reflect the salt sensitivity of unwinding by recBCD enzyme on protein-free DNA, there appears to be little effect of chromatin condensation on unwinding.

Sucrose Gradient Fractionation of Unwinding Reactions Confirms the Fluorometric Results—To demonstrate by an alternate method that the fluorometric assay was measuring the production of ssDNA, the products of an unwinding reaction containing SSB protein were fractionated on neutral sucrose gradients, and the distribution of the DNA was quantified (Fig. 4). Protein-free DNA, in the absence of recBCD enzyme, migrates near the top of the gradient. After reaction with recBCD enzyme, the unwound, SSB protein-coated ssDNA migrates as a diffuse species from the center to the top of the gradient. Native chromatin which is depleted of histone H1 also migrates near the center of the gradient; consequently, this species is difficult to separate from that of the unwound DNA species, although it is apparent that some reaction occurs when recBCD enzyme is present (data not shown). Because the buoyant density of native chromatin containing histone H1 is greater than that of the H1-depleted chromatin, the products of unwinding using this DNA can be distinguished, and they are observed to migrate at the position of the unwound protein-free DNA that is bound by SSB protein (Fig. 4). The similarity of the migration profiles for the unwound DNA in Fig. 4, taken together with the failure of free histones to significantly compete with SSB protein for ssDNA as measured by fluorescence titration, suggests that histones are not associated with the SSB protein-ssDNA complexes detected in the sucrose gradient experiments. Thus, these data argue that chromatin structure is disrupted by recBCD enzyme helicase activity.

DISCUSSION

Reconstituted chromatin DNA containing arrays of regularly spaced, positioned nucleosomes can be unwound by recBCD enzyme, a well-characterized helicase. The rate of unwinding is reduced when nucleosomes are present (Fig. 2), with a maximum inhibition of approximately 60–70% when the template is reconstituted at a histone/DNA weight ratio of 1:1, a ratio which produces templates which are completely bound by histone octamers (27, 29, 42, 43). Experiments using native chromatin isolated from HeLa cell nuclei (Table II; Fig. 4) demonstrate that this physiological template is also capable of being unwound. While the results with the H1-depleted DNA are quantitatively similar to those obtained with highly reconstituted chromatin templates, chromatin substrates containing the linker histone H1 are unwound to a lesser, although measurable, extent.

There are two possible fates for the displaced histone octamers. The dissociated octamers may remain free in solution in equilibrium with H3/H4 tetramers and H2A/H2B dimers. Alternatively, there may be a nonspecific association of various histone assemblies with the unwound ssDNA. Since we observe ~60% quenching at the highest reconstitution ratios, it could be postulated that all of the DNA is unwound and that the histones associate with one strand, while SSB binds only to the other strand. Direct competition experiments between SSB protein and free histone octamers, however, yield 85% of the expected quenching of SSB protein fluorescence, indicating that no more than 15% of the ssDNA produced by unwinding highly reconstituted chromatin (C_n = 0.7) is bound by (or renatured by) histones (data not shown). Silver staining of polyacrylamide gels of sucrose gradient fractions of native chromatin unwinding reactions confirms that, in the presence of recBCD enzyme, free histones are present at the top of the gradient, as expected if they are displaced from the DNA during unwinding.[2] In addition, *in vivo* experiments detect at least partial dissociation of nucleosomes during transcription and replication (44); it is likely that a similar process occurs during unwinding by a helicase. Our results do not necessarily conflict with previous studies which indicated that nucleosomes transfer directly from the region ahead of a transcribing RNA polymerase molecule to the DNA behind the enzyme (10) because in the helicase assay, renaturation of the DNA behind the enzyme is precluded by the binding of SSB protein. Consequently, no duplex DNA acceptor is available for direct transfer of the histone octamer. *In vivo*, however, replication and recombination processes utilize SSB proteins to maintain the transient single-stranded character of the unwound DNA;

[2]T. E. O'Neill, unpublished observation.

thus, our system models those physiological processes.

More significant than the reduction in the rate of unwinding is the decrease in the processivity of helicase activity (Fig. 3). On templates with a moderate to high density of nucleosomes, the processivity of recBCD enzyme is reduced to ≈1.2 kb/end, a value 25-fold less than that observed on protein-free DNA (30 kb/end (22)). Although it might be argued that the observed unwinding occurs only on DNA molecules which are nucleosome-free, this explanation for the reduction in extent is unlikely for two reasons. First, under the conditions used to assemble the chromatin DNA, at a weight ratio of 1:1, the DNA is fully reconstituted with positioned nucleosomes (27, 29, 42, 43). Second, nucleosome cores do not assemble cooperatively onto these repeat sequences (43). Thus, at even low extents of reconstitution, the majority of nucleosome cores should be randomly distributed, and not clustered, on the DNA substrate. One explanation which might be proposed to account for this observation is that the enzyme is incapable of displacing histone octamers, and instead pushes them along the DNA until there is no linker DNA separating neighboring nucleosomes. Assuming this model to be valid, only ~1100 bp (18 positioning sequences × 60 bp of linker DNA/sequence) could be unwound on a fully reconstituted molecule. We detect twice as much unwound DNA, however, suggesting that the histones are instead displaced.

Our finding that unwinding of chromatin DNA does not require dissociation of the histone octamer is consistent with previous data that transcription by T7 RNA polymerase is not affected by extensive cross-linking of the histone octamer (36). Thus, models which propose transient association of a tetramer with ssDNA during transcription (see, for example Ref.41), and presumably during other processes which denature DNA, do not need to be invoked.

While recBCD enzyme is less efficient at unwinding nucleosomal DNA, the precise mechanism by which the rate and processivity of the enzyme's helicase activity are reduced has not been determined. It was possible that the observed unwinding rate on the nucleosomal templates is a composite of two rates: one from the linker DNA between core particles which is similar to that measured on protein-free DNA, and a slower rate of unwinding from the DNA within the core particle. We attempted to confirm this model by separating the products of an unwinding reaction on either agarose or polyacrylamide gels. Time points of an unwinding reaction were treated with S_1 nuclease to degrade the unwound ssDNA tails (under the acidic conditions of the digestion, SSB protein dissociates from the ssDNA and does not interfere with quantitation) (22). If unwinding of the linker DNA is fast relative to that within the core particle and remained synchronous (40), a ladder of bands with a spacing of ~207 bp should be observed. No discrete bands were detected; instead, a disperse population of partially unwound DNA molecules was observed (data not shown), arguing against such a scheme.

Based upon the reduction in rate of unwinding observed on an aliquot of DNA added after the enzyme has been exposed to chromatin DNA (Table I), it appears that some proportion of the enzyme is effectively inactivated. Because inhibition of unwinding is not observed when free histone octamers are added to a reaction, it is unlikely that the displaced histones directly interfere with the helicase activity of the enzyme. A more reasonable explanation for this behavior is that some fraction of the enzyme population becomes either sequestered (in a paused but otherwise active form) or inactivated on the nucleosomal template, or is unable to reinitiate unwinding on a subsequent DNA molecule. Although we have no data to suggest why enzyme dissociation or reinitiation appears to be impaired, recent studies concerning the effect of the recombination hotspot χ on the activities of recBCD enzyme may provide insight into this behavior (45). When recBCD enzyme encounters a χ site (5′-GCTGGTGG-3′), its dsDNA exonuclease activity is attenuated, although it is still able to unwind DNA (40, 46). If the reaction contains ATP concentration in excess of the Mg^{2+} concentration, the altered enzyme is unable to reinitiate unwinding on a subsequent DNA molecule (45). Because this phenomenon is also observed with *in vitro* reconstituted recBC enzyme lacking the recD subunit, it has been proposed that the productive interaction of recBCD enzyme with χ results in functional inactivation or loss of the recD subunit. This modified enzyme is capable of unwinding the DNA molecule with which it is associated but cannot reinitiate unwinding on other DNA molecules (45). All of the experiments reported herein utilized conditions (3 mM ATP, 1 mM $Mg(OAc)_2$) which would maintain the inactivated state if it were to form. Although the 207_{18} DNA fragment lacks a χ sequence, it is possible that the presence of nucleosomes infrequently results in a similar inactivation or dissociation of the recD subunit, thus producing enzyme which is unable to reinitiate unwinding.

Despite the heterologous nature of this system, inferences can be made concerning the *in vivo* applicability of such studies. Within the *E. coli* cell, the chromosomal DNA is assembled into higher order structure by small, basic proteins such as HU and IHF. In addition, sequence-specific DNA-binding proteins such as repressors and other regulatory proteins will be bound to the DNA. Although this problem is typically ignored when *in vitro* studies are performed, it is clear that any global process such as replication, recombination, or repair must deal with these types of nucleoprotein structures. From these results, we propose that one method by which this is accomplished is through the action of a DNA helicase. In *E. coli*, helicases play critical roles in each of these processes, and perhaps one reason for this is that such an activity is required to clear the DNA of other protein·DNA complexes. The *in vitro* biochemistry of these processes as derived from eukaryotic organisms is not as developed, but it is not unreasonable to propose a similar, and perhaps even more critical, role for DNA helicases in these organisms, which contain the additional barrier of highly condensed chromatin.

In some respects, these studies parallel what has been observed in studies of transcription on chromatin templates. Although it was previously believed that once transcription was initiated, subsequent elongation of the transcript was unaffected by the presence of nucleosomes on the gene being transcribed, recent studies indicate an effect on both initiation and elongation by T7 RNA polymerase and RNA polymerase II (35, 47, 48). Likewise, in these unwinding studies, an effect on both the rate of unwinding and its elongation, or processivity, is observed. Unlike results observed with transcription studies, however, we detect unwinding in the presence of condensed chromatin and histone H1. The presence of H1 linker histone has been shown to significantly inhibit transcription, replication, and recombination reactions on chromatin DNA *in vitro* (see, for example, Refs. 13, 49–51). This finding therefore indicates perhaps one means by which this barrier is overcome *in vivo* through the action of a DNA helicase. These results may also explain the difference between *in vivo* and *in vitro* measurements of the processivity of recBCD enzyme (22, 52, 53). Roman *et al.* (22) proposed that this discrepancy (*i.e.* the lower apparent processivity *in vivo*) might be due to the presence of DNA binding proteins on the physiological substrate which would inhibit translocation of the helicase.

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REFERENCES

1. Felsenfeld, G. (1992) *Nature* **355,** 219–224
2. Wolffe, A. (1992) *Chromatin: Structure and Function*, Academic Press, London
3. Haber, J. E. (1992) *Trends Genet.* **8,** 446–452
4. Lorentz, A., Heim, L., and Schmidt, H. (1992) *Mol. Gen. Genet.* **233,** 436–442
5. Ponticelli, A. S., and Smith, G.R. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 227–231
6. White, M. A., Dominska, M., and Petes, T. D. (1993) *Proc. Natl. Acad. Sci. U. S. .A* **90,** 6621–6625
7. Bonne-Andrea, C., Wong, M. L., and Alberts, B. M. (1990) *Nature* **343,** 719–726
8. Almouzni, G., Clark, D. J., Mechali, M., and Wolffe, A. P. (1990) *Nucleic Acids Res.* **18,** 5767–5774
9. Krude, T., and Knippers, R. (1991) *Mol. Cell. Biol.* **11,** 6257–6267
10. Randall, S. K., and Kelly, T. J. (1992) *J. Biol. Chem.* **267,** 14259–14265
11. Clark, D. J., and Felsenfeld, G. (1992) *Cell* **71,** 11–22
12. Ramdas, J., Mythili, E., and Muniyappa, K. (1989) *J. Biol. Chem.* **264,** 17395–17400
13. Ramdas, J., Mythili, E., and Muniyappa, K. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 1344–1348
14. Taylor, A. F. (1988) in *Genetic Recombination* (Kucherlapati, R., and Smith, G. R., eds) pp. 231–263, American Society for Microbiology, Washington, D. C.
15. Smith, G. R. (1990) in *Nucleic Acids and Molecular Biology* (Eckstein, R., and Lilley, D. M. J., eds) Vol. 4, pp. 78–98, Springer-Verlag, Berlin
16. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S.D., and Rehrauer, W. M. (1994) *Microbiol. Rev.* **58,** 401–465
17. Buttin, G., and Wright, M. (1968) *Cold Spring Harbor Symp. Quant. Biol.* **33,** 259–269
18. Wright, M., Buttin, G., and Hurwitz, J. (1971) *J. Biol. Chem.* **246,** 6543–6555
19. Goldmark, P. J., and Linn, S. (1972) *J. Biol. Chem.* **247,** 1849–1860
20. Taylor, A. F., and Smith, G. R. (1980) *Cell* **22,** 447–457
21. Telander-Muskavitch, K. M., and Linn, S. (1982) *J. Biol. Chem.* **257,** 2641–2648
22. Roman, L. J., Eggleston, A. K., and Kowalczykowski, S.C. (1992) *J. Biol. Chem.* **267,** 4207–4214
23. Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2863–2873
24. Eggleston, A. K., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **231,** 621–633
25. Roman, L. J., and Kowalczykowski, S. C. (1989) *Biochemistry* **28,** 2873–2881
26. Simpson, R. T., Thoma, F., and Brubaker, J.M. (1985) *Cell* **42,** 799–808
27. Hansen, J. C., Ausio, J., Stanik, V. H., and van Holde, K. E. (1989) *Biochemistry* **28,** 9129–9136
28. Dong, F., Hansen, J. C., and van Holde, K. E. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5724–5728
29. Meersseman, G., Pennings, S., and Bradbury, E. M. (1991) *J. Mol. Biol.* **220,** 89–100
30. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
31. Eggleston, A. K., and Kowalczykowski, S. C. (1993) *J. Mol. Biol.* **231,** 605–620
32. Eichler, D. C., and Lehman, I. R. (1977) *J. Biol. Chem.* **252,** 499–503
33. LeBowitz, J. (1985) Biochemical Mechanism of Strand Initiation in Bacteriophage Lambda DNA Replication, Ph.D. Thesis, The Johns Hopkins University, Baltimore, MD
34. Ruyechan, W. T., and Wetmur, J. G. (1976) *Biochemistry* **15,** 5047–5064
35. O'Neill, T. E., Roberge, M., and Bradbury, E. M. (1992) *J. Mol. Biol.* **223,** 67–78
36. O'Neill, T. E., Smith, J. G., and Bradbury, E. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 6203–6207
37. Taylor, A. F., and Smith, G. R. (1985) *J. Mol. Biol.* **185,** 431–443
38. Ganesan, S., and Smith, G. R. (1993) *J. Mol. Biol.* **229,** 67–78
39. Karu, A. E., Mackay, V., Goldmark, P. J., and Linn, S. (1973) *J. Biol. Chem.* **248,** 4874–4884
40. Dixon, D. A., and Kowalczykowski, S. C. (1993) *Cell* **73,** 87–96
41. van Holde, K. E., Lohr, D. E., and Robert, C. (1992) *J. Biol. Chem.* **267,** 2837–2840
42. Allen, M. J., Dong, X. F., O'Neill, T. E., Yau, P., Kowalczykowski, S. C., Gatewood, J., Balhorn, R., and Bradbury, E. M. (1993) *Biochemistry* **32,** 8390–8396
43. Hansen, J. C., and Lohr, D. (1993) *J. Biol. Chem.* **268,** 5840–5848
44. Jackson, V. (1990) *Biochemistry* **29,** 719–731
45. Dixon, D. A., Churchill, J. C., and Kowalczykowski, S. C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2980–2984
46. Dixon, D. A., and Kowalczykowski, S. C. (1991) *Cell* **66,** 361–371
47. Izban, M. G., and Luse, D. S. (1991) *Genes & Dev.* **5,** 683–696
48. Kirov, N., Tsaneva, I., Einbinder, E., and Tsanev, R. (1992) *EMBO J.* **11,** 1941–1947
49. Shimamura, A., Sapp, M., Rodriguez-Campo, A., and Worcel, A. (1989) *Mol. Cell. Biol.* **9,** 5573–5584
50. Takada, S., Magira, T., and Yamamura, M. (1989) *Biochem. Biophys. Res. Commun.* **160,** 711–714
51. Laybourn, P. J., and Kadonaga, J. T. (1991) *Science* **254,** 238–245
52. Cheng, K. C., and Smith, G. R. (1989) *Genetics* **123,** 5–17
53. Ennis, D. G., Amundsen, S. K., and Smith, G. R. (1987) *Genetics* **115,** 11–24