DNA-dependent protein kinase (DNA-PK) is involved in joining DNA double-strand breaks induced by ionizing radiation or V(D)J recombination. The kinase is activated by DNA ends and composed of a DNA binding subunit, Ku, and a catalytic subunit, DNA-PKCS. To define the DNA structure required for kinase activation, we synthesized a series of DNA molecules and tested their interactions with purified DNA-PKCS. The addition of unpaired single strands to blunt DNA ends increased binding and activation of the kinase. When single-stranded loops were added to the DNA ends, binding was preserved, but kinase activation was severely reduced. Obstruction of DNA ends by streptavidin reduced both binding and activation of the kinase. Significantly, short single-stranded oligonucleotides of 3–10 bases were capable of activating DNA-PKCS. Taken together, these data indicate that kinase activation involves a specific interaction with free single-stranded DNA ends. The structure of DNA-PKCS contains an open channel large enough for double-stranded DNA and an adjacent enclosed cavity with the dimensions of single-stranded DNA. The data presented here support a model in which duplex DNA binds to the open channel, and a single-stranded DNA end is inserted into the enclosed cavity to activate the kinase.

Cells recognize and respond to a multitude of different DNA lesions by activating pathways for apoptosis, cell cycle arrest, or DNA repair. Little is known about how DNA lesions are recognized and transduced into a signal for these cellular responses. In the case of DNA double-strand breaks (DSBs) induced by ionizing radiation, recognition is critically important, because DSBs can lead to chromosomal fragmentation and cell death, or to chromosomal translocations and cancer.

Ionizing radiation activates the c-Abl tyrosine kinase, which has undefined physiological functions (1, 2). Ionizing radiation also activates the ATM kinase and DNA-dependent protein kinase (DNA-PK), which have homologous kinase domains. ATM phosphorylates p53 to induce cell cycle arrest or apoptosis (3). DNA-PK is required for the repair of DSBs produced by ionizing radiation and V(D)J recombination, the process that generates immunological diversity in antibodies and T cell receptors (4). Understanding how DNA-PK is activated by DSBs can establish a paradigm for how proteins signal the presence of DNA lesions.

DNA-PK is a serine-threonine protein kinase consisting of DNA binding and catalytic subunits. The DNA binding subunit is the Ku protein, a heterodimer of 70 and 86 kDa that binds to DNA ends, nicks, and structures containing a transition fork between double-stranded DNA and two single strands (5–9). The catalytic subunit of DNA-PK (DNA-PKCS) is a 465-kDa polypeptide (10) that is sufficient for the kinase activity of the enzyme (11–13). DNA-PKCS is recruited for activation at DNA ends by Ku at physiological salt concentrations (14, 15), but the kinase is fully activated by DNA ends in the absence of Ku at low salt concentrations (12).

Several lines of evidence indicate that DNA-PK is involved in the cellular response to DSBs. The otherwise latent kinase activity of DNA-PK is activated by DNA ends (14, 16). The catalytic domain of DNA-PKCS is mutated in the severe combined immunodeficiency mouse (17, 18), which is defective in the repair of DSBs (19–21). Additional studies have suggested that the catalytic kinase activity of DNA-PK is required for rejoining DSBs both in intact cells (22) and in a cell free system (23).

A number of DNA structures have been tested for their ability to activate DNA-PK. DNA with blunt ends, 5′ overhanging ends, or 3′ overhanging ends activate DNA-PK with equal efficiency (14), whereas double-stranded DNA with hairpin ends fails to activate the kinase (24). Superoiled plasmid DNA fails to activate DNA-PK, but supercoiled plasmid DNA containing the NRE1 sequence from mouse mammary tumor virus was reported to activate the kinase (25, 26). Based on these studies, it was not clear what specific DNA structure was critical for the activation of DNA-PK.

In these earlier studies, the DNA structures were tested with different enzyme preparations and a variety of protein substrates. Interpretations of the results were potentially confounded by several factors: DNA preparations may have contained contaminating DNA structures, and enzyme preparations often included Ku, which may have altered or obscured properties of the DNA structures upon binding. Therefore, to define precisely the DNA structure required for kinase activation, we undertook a systematic study of a homogeneous preparation of DNA-PKCS with a series of gel-purified...
DNA structures in the absence of any cofactors or contaminating proteins.

**MATERIALS AND METHODS**

**Oligonucleotides and Plasmids—**Oligonucleotides were purified on oligonucleotide purification cartridges (Poly-Pak™ cartridge, Glen Research) to remove truncated synthesis products. The oligonucleotides synthesized as oligo(dT) were further purified on DNA-Pak HPLC columns (Dyna1) using a NaClO gradient in 25 mM Tris, pH 7.4, 5% acetonitrile, desalted on Sep-Pak columns (Waters), lyophilized, and dissolved in TE buffer (10 mM Tris-HCl, pH 7.4, 0.5 mM EDTA).

Double-stranded DNA fragments were made by annealing complementary oligonucleotides in a 1:1 molar ratio in TE buffer and separated from single-stranded DNA on 12% non-denaturing polyacrylamide gels, which were then stained with ethidium bromide. DNA structures in the absence of any cofactors or contaminants were confirmed by agarose gel electrophoresis in the presence of ethidium bromide, electroelution, and reconfirmed on a sequencing gel (7 M urea, 40% formamide, 1× TBE buffer run at 65 °C), and analyzed by autoradiography (Fig. 2). The cleavage products migrated with the expected mobilities as outlined in Fig. 2A, confirming the structure and purity of the hairpin preparation.

The DNA fragments ended in single-stranded DNA loops were generated and purified by methods similar to those used for the DNA fragment with hairpin ends. The f49NRE1-sL5 fragment, which ends with 5 base single-stranded DNA loops of dT and contains the NRE1 sequence, was used to construct the f32-sL5 fragment, which ends with a 5-base single-stranded DNA loop (oligo74: 5'-ATGGTGGACGGCGTAGACGCG-3'). Three oligonucleotides described in this paper, unpaired nucleotides are in brackets. The blunt-ended single-stranded DNA fragment from Fig. 2A was then labeled with [γ-32P]ATP as described (27).

**Purification of DNA-PKcs and Ku—**DNA-PKcs and Ku were purified from placenta as described (12). To omit multiple freeze-thaw cycles, which severely degraded specific activity of the enzyme, DNA-PKcs and Ku were kept in storage buffer (55% glycerol, 250 mM NaCl, 2.5 mM EDTA, 25 mM Tris-HCl, pH 7.4, 5 mM dithiothreitol, 10 mM 2-mercaptoethanol, 0.2 mg/ml bovine serum albumin, and a mixture of protease inhibitors) at -20 °C. From stock buffer, a slow decrease in kinase activity was detected, whereas no effect on the ability of Ku to bind DNA could be detected even after several freeze-thaw cycles. To ensure that our results were not compromised by this effect, we assessed the potential effect of autophosphorylation under our experimental conditions, a slow decrease in kinase activity was detected, whereas no effect on the ability of Ku to bind DNA could be detected even after several months. For long-term storage, DNA-PKcs and Ku were kept in Buffer B (5% glycerol, 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 250 mM NaCl, 10 mM 2-mercaptoethanol, and a mixture of protease inhibitors) at -80 °C (12).

**Kinase Assay—**Kinase activity was measured in 10 μl of kinase buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 20 mM MgCl2, 2 mM dithiothreitol, 10 mM 2-mercaptoethanol, 5% glycerol, 62.5 μM ATP) supplemented with 0.5 mg/ml substrate peptide and [γ-32P]ATP as described (27). Phosphate transfer to the substrate peptide was calculated and expressed as mol of phosphate transferred per min of DNA-PKcs per μg (mol PO4/mg DNA-PKcs/min). Reaction times were adjusted so that less than 10% of the substrate peptide and ATP was consumed during the kinase reaction. All kinase assays were performed at least twice. Although storage led to some loss of enzymatic activity, there were no significant variations in relative kinase activities for different DNA substrates when experiments were repeated.

Chan et al. (28) reported that DNA-PK auto-phosphorylation was associated with inactivation under some conditions, but not when peptide analogues or nucleotides were present in the kinase assay, so that our results were not compromised by this effect, we assessed the potential effect of auto-phosphorylation under our experimental conditions. Preincubation of DNA-PKcs with peptide and ATP resulted in readily detectable auto-phosphorylation, but this auto-phosphorylation did not have a detectable effect on subsequent activity of the kinase.

**Electrophoretic Mobility Shift Assay (EMSA)—**EMSAs were performed in kinase buffer with ATP and 0.5 mg/ml bovine serum albumin ligated, nicked DNA fragment. (Compare hairpin to nicked hairpin in Fig. 2B.)

Bands corresponding to the hairpin and the nicked hairpin were cut out of the gels, electroeluted, and purified as described (27). To confirm that the purified hairpin preparation was free of aberrant ligation products, we adopted a sequential electrophoresis and autoradiography approach. The hairpin preparation was analyzed on a 12% non-denaturing polyacrylamide gel and stained with ethidium bromide (see Fig. 2C). As expected, the putative hairpin-ended DNA fragment migrated with the same mobility as open-ended DNA fragments of 44 bp. To further confirm that the putative hairpin had the expected structure and was free of nicks, the putative hairpin and nicked hairpin preparations were incubated with a panel of restriction enzymes. The cleavage products were then labeled with [γ-32P]ATP using the exchange reaction for T4 polynucleotide kinase, resolved on a denaturing polyacrylamide sequencing gel (7 μ urea, 40% formamide, 1× TBE buffer run at 65 °C), and analyzed by autoradiography (see Fig. 2D). The cleavage products migrated with the expected mobilities as outlined in Fig. 2A, confirming the structure and purity of the hairpin preparation.
Activation of DNA-PK by Single-stranded DNA Ends

RESULTS

Unpaired Single-stranded DNA Ends Increase the \( V_{\text{max}} \) for DNA-PK<sub>Cs</sub> Activation—We previously reported that a 12-bp DNA fragment failed to activate the kinase significantly, but when 5 bases of unpaired single strands were added to both ends of each strand, the kinase was strongly activated (27). To explore the effect of unpaired single-stranded ends in greater detail, we constructed a series of symmetrical DNA fragments derived from the 12-bp DNA fragment (f12) by adding unpaired single strands at different ends (Fig. 1). To avoid the formation of secondary structures, the single strands consisted of 5 bases of dT. The single strands were added to f12 at its 3′ ends (f12-ss3′), 5′ ends (f12-ss5′), or both 3′ and 5′ ends (f12-ss3′5′). \( V_{\text{max}} \) was determined from the best fit to the Michaelis-Menten equation. The \( V_{\text{max}} \) values were as follows (in mol PO<sub>4</sub>/mol DNA-PK<sub>Cs</sub>/min): f22-ss3′, 470; f12-ss3′, 300; f22, 230; f12-ss5′, 85; and f12, too low to be determined.

Addition of unpaired single strands to the 5′ ends of f12 (f12-ss5′) produced a moderate increase in \( V_{\text{max}} \). Addition of unpaired 3′ ends (f12-ss3′) produced a larger increase in \( V_{\text{max}} \), more than 3-fold greater than that for unpaired 5′ ends. In fact, the \( V_{\text{max}} \) for f12-ss3′ was slightly but reproducibly higher than the \( V_{\text{max}} \) for f22, which is equivalent to the highest \( V_{\text{max}} \) for blunt-ended DNA of any length (27). Addition of unpaired single strands at both 3′ and 5′ ends (f12-ss3′5′) resulted in even more efficient activation, with a \( V_{\text{max}} \) more than 2-fold higher than the \( V_{\text{max}} \) for f22, as we reported previously (27). Therefore, DNA-PK<sub>Cs</sub> was activated most efficiently when both 3′ and 5′ ends were present.

Unpaired Single-stranded DNA Ends Increase DNA Binding to DNA-PK<sub>Cs</sub>—The relative binding affinities of different DNA substrates for DNA-PK<sub>Cs</sub> can be estimated from the steady-state kinetics of kinase activation as a function of DNA concentration. At the low salt concentration of the kinase buffer used in Fig. 1, the concentrations of f22, f12-ss3′, and f12-ss3′5′ that produced half maximal kinase activity (\( V_{\text{max}}/2 \)) were equal to or lower than the enzyme concentration (1.9 nM).

Table I

| DNA | \( K_m \) | \( V_{\text{max}} \) | \( R \) |
|-----|--------|--------|-----|
| f12 | ND     | ND     | 0.59 |
| f12-ss5′ | 48     | 59     | 0.97 |
| f12-ss3′ | 32     | 170    | 0.98 |
| f12-ss3′5′ | <2     | 370    | 0.98 |
| f22 | 12     | 150    | 0.99 |

This meant that most of the DNA was bound to the enzyme, and the \( K_m \) could not be determined reliably.

Attempts to decrease the enzyme concentration resulted in an unacceptable loss of kinase activity. On the other hand, the binding of DNA to DNA-PK<sub>Cs</sub> is destabilized by salt (12). Therefore, to obtain a better estimate of both \( K_m \) and \( V_{\text{max}} \) for each DNA substrate in a single buffer system, we supplemented the kinase buffer with 25 mM NaCl (Table I). Although this buffer system required significantly higher DNA concentrations to reach \( V_{\text{max}} \), we were able to generate data to fit the Michaelis-Menten equation. The \( V_{\text{max}} \) for each DNA substrate was slightly lower than that found for the low salt buffer, but their relative values were preserved (compare Table I and Fig. 1).

Assuming that binding of DNA to enzyme was rate-limiting for kinase activity, \( K_m \) reflected the apparent dissociation constant \( K_{\text{d}} \) between DNA and DNA-PK<sub>Cs</sub>. The \( K_m \) for unpaired 3′ ends (f12-ss3′) was approximately equal to that for unpaired 5′ ends (f12-ss5′), despite a nearly 3-fold greater \( V_{\text{max}} \). Thus, the enzyme showed no clear preference for binding to unpaired 3′ ends over unpaired 5′ ends, although it showed a significant increase in activation by 3′ ends once binding was complete. Strikingly, the \( K_m \) for DNA fragments with unpaired single strands at both 3′ and 5′ ends (f12-ss3′5′), was less than \( 1/5 \) the \( K_m \) for f12-ss3′ or f12-ss5′ and less than \( 1/3 \) the \( K_m \) for a blunt-ended duplex DNA containing the same total number of bases, f22. Furthermore, the \( V_{\text{max}} \) for f12-ss3′5′ was more than 2-fold greater than for f12-ss3′ and f22 and more than 6-fold higher than for f12-ss5′. These results suggest that the enzyme binds productively to DNA in a conformation that utilizes both 3′ and 5′ single-stranded ends.

Single-stranded Loops or Hairpins at DNA Ends Severely Reduce DNA-PK<sub>Cs</sub> Activation—Because unpaired single-stranded DNA ends increased kinase activation, we wondered whether the increase was due to unpaired DNA or to single-stranded DNA ends. DNA fragments were constructed with ends consisting of a covalent DNA hairpin or a single-stranded DNA loop. Construction of these molecules utilized oligonucleotides that contained self-complementary sequences. Annealing occurred by both intramolecular and intermolecular reactions, and the ligation products were a complex mixture of different molecules produced by different annealing reactions and different degrees of ligation. Thus, special care was required to purify the DNA fragments with hairpin ends and single-stranded DNA loops (see under “Materials and Methods”).
to verify that the purified preparation was free of contaminating DNA molecules (Fig. 2).

Three molecules with covalently closed DNA ends were constructed: a 44-bp hairpin-ended DNA fragment (f44-H), a 32-bp DNA fragment with a single-stranded loop of 5 bases of dT at each end (f32-ss3), and a 32-bp DNA fragment with a single-stranded loop of 10 bases of dT at each end (f32-ssL10). The molecules were designed to be approximately matched in overall length (Fig. 3).

The DNA molecules with hairpin ends and single-stranded loops activated DNA-PKCS only slightly over background. This small degree of activation was reproducible and not due to a small amount of contaminating open-ended DNA, because activation reached $V_{max}$ at relatively low DNA concentrations. The inefficiency of activation was not due to the presence of contaminating inhibitors, because restriction enzyme cleavage of the molecules resulted in robust activation of the kinase (data not shown). Furthermore, activation by DNA with single-stranded loops was inefficient for NaCl concentrations from 0 to 100 mM and Mg$^{2+}$ and Mn$^{2+}$ concentrations from 5 to 20 mM (data not shown).

Contrary to our results, Morozov et al. (29) previously reported that DNA with single-stranded loops efficiently activated DNA-PK. However, no protocol for purification of the DNA substrate was described. In fact, purification of DNA substrate was of vital importance, because we found that aberrant products were formed with high efficiency when self-complementary oligonucleotides were annealed and ligated. Indeed, DNA-PKCS was activated by partially purified f32-ssL10 preparations.

Significantly, f32-ss3/5’ was 30-fold more potent than f32-ssL10 in activating DNA-PK$_{CS}$, even though the two molecules are identical except for the presence or absence of single-stranded ends. These results demonstrate that DNA molecules containing hairpin ends or ends with single-stranded loops fail to activate the kinase efficiently. In fact, they suggest that full kinase activation requires interaction with free single-stranded DNA ends.

**DNA Ends with Single-stranded Loops Bind Efficiently to DNA-PK$_{CS}$**

Because the addition of hairpins or single-stranded loops to DNA ends severely reduced kinase activation, we wished to determine whether this reduction was due to loss of binding to the enzyme. The DNA fragments in Fig. 3 were compared for their ability to compete for binding to a labeled blunt-ended DNA fragment (f42) in an EMSA (12). In this assay, labeled f42 and unlabeled competitor DNA were mixed together and then incubated with DNA-PK$_{CS}$ in kinase buffer. The resulting protein-DNA complexes were resolved by non-denaturing polyacrylamide gel electrophoresis and analyzed by autoradiography and phosphorimaging. The concentration of competitor DNA causing 50% inhibition of binding to f42 (IC$_{50}$) was calculated by fitting the phosphorimager data to simple competitive inhibition kinetics. Comparison of the IC$_{50}$ for different competitor DNAs was used as a measurement of relative binding affinities.

The EMSA was used to measure binding of DNA-PK$_{CS}$ (Fig. 4) to the DNA fragments in Fig. 3 and to plasmid DNA. The DNA fragment with unpaired single-stranded DNA at 3’ and 5’ ends (f32-ss3/5’) was the most active competitor (IC$_{50}$ = 7 ng/ml), more than 4-fold more potent than blunt-ended DNA, f42 (IC$_{50}$ = 30 ng/ml). This confirms the conclusion from Table 1 that the presence of unpaired single strands stimulates binding of DNA to DNA-PK$_{CS}$. Although DNA with ends consisting of single-stranded loops (f32-ssL10) was unable to activate...
DNA-PKCS, it competed for binding to DNA-PKCS (IC₅₀ = 20 ng/ml) slightly more effectively than f42. Hairpin-ended DNA (f44-H) competed for binding to DNA-PKCS (IC₅₀ = 300 ng/ml) about 10-fold less effectively than f42. Nevertheless, the competition by f44-H was significant when compared with competition by relaxed plasmid DNA (data not shown) or supercoiled plasmid DNA (IC₅₀ = 12,800 ng/ml). This was consistent with our previous report that hairpin-ended DNA binds but fails to efficiently activate the DNA-PK holoenzyme.

We conclude that DNA-PKCS binds effectively to DNA ending in single-stranded loops, despite failing to activate the kinase. The transition fork in f32-ssL10 from double-stranded DNA to two single strands appears to be a critical determinant for effective binding to the enzyme, because hairpin-ended DNA, which lacks such a transition fork, was 1/10 as effective as a competitor for binding. Thus, DNA-PKCS binds effectively to DNA containing a transition fork from double-stranded DNA to two single strands but is activated only if the ends of single-stranded DNA are present.

Blocking DNA Ends with Streptavidin Interferes with Binding and Activation of DNA-PKCS—Our experiments suggested that in order to be activated efficiently, DNA-PKCS must interact with the ends of single-stranded DNA. Modification of the ends to include 5'-phosphates had no effect on kinase activation (data not shown). To further investigate the structural requirements of the single-stranded ends, we constructed a DNA fragment with all four ends modified with biotin on the terminal nucleotide (f32-ss3' B/5' B) (Fig. 5A). Biotin modification by itself had no effect on the ability of the DNA to activate the kinase in the absence of streptavidin.

The biotinylated DNA f32-ss3' B/5' B was then mixed with increasing concentrations of streptavidin and tested for kinase activity. Streptavidin consists of a tetramer of four identical 15-kDa subunits, and it binds tightly to biotin. Addition of streptavidin produced an 80% inhibition of kinase activity. This inhibition was due to a decrease in Vₘₐₓ for the kinase, because the DNA was present in supersaturating concentration (20 nM). Inhibition required streptavidin bound to DNA, because no inhibition was observed when streptavidin was added to DNA lacking biotin (f32-ss3' B/5' B) or when streptavidin binding to the biotinylated DNA was blocked by excess free biotin.

FIG. 3. DNA ends consisting of single-stranded loops or hairpins fail to efficiently activate DNA-PKCS. DNA-PKCS (1.9 nM) was incubated with different DNA molecules in kinase buffer. The experiments included three molecules with covalently closed DNA ends: a 44-bp hairpin-ended DNA fragment (f44-H), a 32-bp DNA fragment with a single-stranded loop of 5 bases at each end (f32-ssL5), and a 32-bp DNA fragment with a single-stranded loop of 10 bases at each end (f32-ssL10). For comparison, the experiments also included two homologous molecules with open DNA ends: a 32-bp DNA fragment with blunt ends (f32) and a 32-bp DNA fragment with 5 bases of single-stranded DNA at the 3' and 5' ends (f32-ss3'/5'). Vₘₐₓ was determined from the best fit to the Michaelis-Menten equation. The Vₘₐₓ values were as follows (in mol PO₄/mol DNA-PKCS/min): f32-ss3'/5', 230; f32, 110; f32-ssL5, 13; f32-ssL10, 8; and f44-H, 8.

FIG. 4. DNA ends with single-stranded loops bind efficiently to DNA-PKCS. A labeled 42-bp blunt-ended DNA fragment (f42) (1 nM, 30 ng/ml) was mixed with competitor DNA in 2-fold increments of concentration and then incubated with DNA-PKCS (2.1 nM) in kinase buffer. The resulting protein-DNA complexes were resolved by nondenaturing gel electrophoresis and visualized by autoradiography (top panel). Complex formation was displayed graphically (bottom panel) after quantification by phosphorimager of the radioactivity in the protein-DNA complexes (migrating at the position marked DNA-PKCS and retained in the well of the gel). The free (unbound) DNA migrated at the position marked F.
We further explored the effect of streptavidin binding to DNA by constructing a series of DNA fragments with biotinylated nucleotides at different positions (Fig. 5B). In these experiments, the DNA concentration was decreased to 1.25 nM, a nearly saturating concentration for f32-ss3/5'. Under these conditions, activation of the kinase by DNA with biotin at all four ends (f32-ss3/B/5'B) was inhibited 95% by adding streptavidin. This nearly complete inhibition allowed us to explore the more subtle effects produced by placing biotin at other positions.

When biotin was added to the DNA fragment at the four

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internal positions adjacent to the unpaired single strands (F2B-ss3’/5’), kinase inhibition still occurred with the addition of streptavidin, but the effect was significantly less pronounced. When the biotin was placed on only the 3’ ends (F2B-ss3’/B/5’) or only on the 5’ ends (F2B-ss3’/B/5’), inhibition by streptavidin was even weaker. Interestingly, obstruction of the 3’ end produced a consistently larger effect than obstruction of the 5’ end. This was consistent with our finding that DNA-PK CS was activated more efficiently by its interaction with 3’ ends (Fig. 1).

To determine whether blocking DNA ends with streptavidin had an effect on binding to DNA-PK CS, we tested the DNA for its ability to compete with unmodified DNA for binding to DNA-PK CS in an EMSA. Unlabeled biotinylated DNA (F2ss3’/B/5’) was tested for its ability to compete with labeled unmodified DNA (F2B) for binding to DNA-PK CS (Fig. 6A). In the absence of streptavidin, the biotinylated DNA competed effectively for binding, as expected. Upon addition of streptavidin, the biotinylated DNA was 1/10 as effective.

The Vmax concentration (25 μM), oligo(dT) activated DNA-PK CS as a function of DNA concentration. Special precautions were taken to remove organic impurities that inhibited the kinase (see under “Materials and Methods”). Presence of these impurities was evident by a decline in kinase activity as the concentration of less purified dT5 preparations increased. Successful removal of the impurities was supported by absence of a decline in kinase activity as the concentration of the fully purified dT5 preparation increased from 10,000 to 50,000 nM (Fig. 7B).

The Vmax for dT5 was 30% of that obtained with the 32-pb blunt-ended DNA fragment F2B. Kinase activity reached half-maximal levels at a DNA concentration of 7200 nM for dT5 and 15 nM for F2B. Thus, dT5 bound to DNA-PK CS with an affinity at least 3 orders of magnitude lower than that for double-stranded F2B. Activation by dT5 was not due to contamination of the preparation with double-stranded DNA, because activation was abolished by digestion of dT5 to mononucleotides with the single-strand specific nuclease S1. When NaCl was added to the kinase buffer at concentrations of 50 and 100 mM, activation by dT5 became completely undetectable, even though activation by F2B was only partially suppressed (data not shown). Thus, activation of DNA-PK CS by dT5 was more salt-sensitive than activation by F2B, consistent with a weaker interaction between DNA-PK CS and dT5. Nevertheless, once dT5 was bound to the enzyme, it was capable of activating DNA-PK CS to significant levels.

We previously reported that activation of DNA-PK CS by double-stranded DNA was inhibited by the single-stranded DNA dT30 (27). We found that the activation of DNA-PK CS by dT5 was similarly inhibited by dT30 (Fig. 7B). This lends further support to the idea that DNA-PK CS is activated by a specific interaction with short single-stranded DNA.

The NRE1 DNA Sequence Is Not Sufficient to Fully Activate DNA-PK CS—Giffen et al. (25, 26) have reported that the NRE1 (negative regulatory element 1) sequence from the mouse mammary tumor virus long terminal repeat binds Ku and activates DNA-PK in the absence of DNA ends. This observation raises the possibility that a specific DNA sequence might be an exception to our conclusion that single-stranded DNA ends are necessary for efficient activation of DNA-PK CS.

To examine whether the NRE1 sequence activates DNA-PK CS under our experimental conditions, we constructed a DNA fragment containing the NRE1 sequence and terminating the single-stranded DNA loops (f49NRE1-ssL5). Compared with DNA with blunt ends (F2B), the f49NRE1-ssL5 DNA failed to efficiently activate DNA-PK CS in either the absence or the presence of Ku (Fig. 8A). In fact, addition of the NRE1 sequence provided no increase over the limited activation observed with the homologous DNA fragment lacking the sequence (F2B-ssL5). Failure to activate the kinase was not due to the presence of inhibitors in the f49NRE1-ssL5 DNA preparation, because efficient activation was observed when the DNA was cleaved with BamHI.

Because Giffen et al. (25, 26) tested the NRE1 sequence in the context of plasmid DNA, we cloned the NRE1 sequence into the pBluescript plasmid. The resulting plasmid (pNRE1) also failed to activate DNA-PK CS (Fig. 8B). Failure to activate was not due to inhibitors in the pNRE1 DNA preparation: pNRE1 cleaved with BamHI was as efficient as F2B in activating DNA-PK CS.
PKCS when Ku was present. In the absence of Ku, pNRE1 cleaved with BamHI failed to activate DNA-PKCS efficiently, because effective recruitment of DNA-PKCS to the end of a long DNA fragment requires Ku (12). Thus, the NRE1 sequence failed to activate DNA-PKCS under the experimental conditions used in this study.

**DISCUSSION**

**Binding of DNA-PKCS to DNA Ends Occurs at a Transition Fork between Double-stranded DNA and Two Single Strands**—To define the structure in DSBs that activates DNA-PKCS, we synthesized a series of DNA fragments with different end structures. The binding of DNA-PKCS to different DNA ends was measured by either of two methods: enzyme kinetics to determine an effective dissociation constant $K_d$ and competitive EMSAs to assess relative binding affinity. When unpaired single strands were added to a 12-bp DNA fragment at the 3' end (f12-ss3') or at the 5' end (f12-ss5'), the polarity of the unpaired single strand did not have a significant effect on the dissociation constant $K_d$. On the other hand, for a DNA fragment containing both 3' and 5' unpaired ends (f12-ss3'/5'), the $K_d$ was less than 1/5 the $K_d$ for f12-ss3' or f12-ss5' and less than 1/6 the $K_d$ for f22, a blunt-ended DNA fragment with the same total number of bases as f12-ss3'/5'. These data suggest that binding of DNA to DNA-PKCS involves melting of the end to form a transition fork between double-stranded DNA and two single strands. Thus, when the transition fork was preformed, part of the binding energy was not utilized to melt the DNA end, and binding increased.

In further support of this hypothesis, a competitive EMSA showed that DNA with unpaired ends (f32-ss3'/5') bound to DNA-PKCS much more effectively than DNA with blunt ends. DNA with ends consisting of single-stranded loops (f32-ssL10) failed to activate the kinase, despite strong binding of f32-ssL10 to DNA-PKCS. To further explore the importance of free DNA ends, we synthesized DNA fragments in which the ends were obstructed by streptavidin. When streptavidin obstructed the single-stranded ends of the DNA fragment (f32-ss3'B/5'B), both kinase activation and binding to DNA-PKCS were strongly inhibited.

The foregoing experiments demonstrated that DNA must contain free single-stranded ends to efficiently activate the kinase. We then tested whether free single strands alone were sufficient for kinase activation. Short single-stranded oligonucleotides were able to significantly activate DNA-PKCS. Nevertheless, activation by the oligonucleotides required very high concentrations of DNA, and maximal activation was 30% that of double-stranded DNA. A possible explanation is that full activation of the kinase requires interactions with both double and single-stranded DNA. Alternatively, activation may occur solely through an interaction with single-stranded DNA, but full activation requires correct presentation of the single-strand in the context of a double-stranded DNA end.

**Structural Model for How DNA Ends Activate DNA-PKCS**—Single molecule cryo-electron microscopy suggests the presence of an enclosed cavity within the DNA-PKCS molecule (30). Electron microscopy of two-dimensional DNA-PKCS crystals reveals three openings to the cavity, each with the dimensions of single-stranded DNA (27). One opening is adjacent to an
DNA-PKCS is shown in the context of interactions between the enzyme DNA enhance kinase activation, because they are more easily threaded into the enclosed cavity (Fig. 9). When threading of the ends reaches a critical length, thus allowing openings of the enclosed cavity. The kinase is inactivated (kinase off) when an unpaired single strand is threaded into one of two openings of the enclosed cavity. The kinase is activated (kinase on) when the single-stranded DNA binding site may lie outside the enclosed cavity. For example, the streptavidin tetramer is 45 × 55 Å (31), and duplex DNA is 24 Å in diameter, whereas the cavity openings are 8 × 16 Å.

Fig. 9B shows the model for DNA-PKCS interacting with DNA in the context of DSB repair. Upon recruitment of DNA-PKCS to the DNA ends by Ku, the model shows DNA bound to the open channel. The ends are then brought into synapsis by a mechanism, mediated in part by Ku (32) and perhaps by DNA-PKCS (33). Once synapsis occurs, the model proposes that DNA-PKCS is activated when a single-stranded end is inserted into the enclosed cavity. One single-stranded end may be threaded into the opening to the cavity adjacent to the open channel. The other single-stranded end may be threaded into an opening on the opposing DNA-PKCS molecule, further stabilizing synapsis of the two DNA ends. A contribution from both single-stranded ends is consistent with the 2-fold increase in \( V_{\text{max}} \) for the kinase when unpaired 5’ ends are added to a DNA molecule with unpaired 3’ ends (Fig. 1).

Nicked DNA fails to activate DNA-PKCS efficiently (24), even though a DNA nick contains two single-stranded ends that potentially activate the kinase. Perhaps the kinase is not activated because the two single-stranded ends at a DNA nick are topologically different from the single-stranded ends at a DSB. It is also possible that DNA-PKCS activation may require synapsis of a second DNA-PKCS molecule (34), and the synaptic configuration depicted in Fig. 9B cannot form at a DNA nick.

Deletions in the coding joints from V(D)J recombination suggest that end-processing is capable of generating unpaired single strands (35, 36). When a single-stranded end becomes too long, the kinase is inhibited. For example, dT30 inhibits activation of the kinase by double-stranded DNA (27). Oligo(dT) and DNA ends with unpaired single strands are progressively less effective in activating the kinase as the single strands increase beyond a length of 10 bases (Fig. 7, and data not shown). In the model, kinase activity and associated end-processing cease when an unpaired single strand penetrates deeply into the cavity.

To complete the end-joining reaction, complementary regions of microhomology in single strands from opposing ends are annealed to each other, perhaps requiring a RecA-like protein. Unpaired single strands are removed by an exonuclease or flap-endonuclease. The remaining nicks are ligated by the XRCC4/ligase IV complex (37), leaving intact DNA with a deletion extending to the microhomology regions. Thus, the model explains how deletions occur in the coding joints after V(D)J recombination. Regulation of the kinase by the processed single-stranded ends limits the size of the deletions.

Activation of DNA-PKCS in the Absence of DNA Ends—Although we have demonstrated that DNA-PKCS is activated efficiently by single-stranded DNA ends, the kinase may be activated by other mechanisms in the absence of DNA ends. Giffen et al. (25, 26) reported that Ku recruits DNA-PKCS specifically to the NRE1 sequence, which is present in the long terminal repeat of mouse mammary tumor virus and located near the binding site for the glucocorticoid receptor. Upon assembly at the NRE1 sequence, DNA-PK phosphorylates the glucocorticoid receptor and represses transcription. To assess the effect of the NRE1 sequence in the context of our experimental system, we placed the NRE1 sequence in a DNA fragment ending in single-stranded loops and in a supercoiled plasmid. In both cases, the NRE1 sequence failed to enhance kinase activation beyond low levels in our assay, which measured phosphorylation of a free peptide. By contrast, Giffen et al.
(25, 26) measured phosphorylation of the glucocorticoid receptor, which was bound to a DNA sequence adjacent to the NRE1 sequence. Thus, the NRE1 sequence may recruit DNA-PK for phosphorylation of the co-localized glucocorticoid receptor, even though the level of kinase activity is too low for efficient phosphorylation of free peptide.

Yavuzer et al. (38) reported that the high affinity DNA-binding protein C1D interacts with DNA-PKCS. Furthermore, C1D associated with supercoiled DNA can direct the phosphorylation of free peptide. Because the kinase would be only minimally activated.

This effect appears to require precise juxtaposition of the target sequence. Thus, the NRE1 sequence may recruit DNA-PK for phosphorylation of free peptide. Despite the absence of free DNA ends, C1D in association with supercoiled DNA is able to activate DNA-PK to a level comparable to that produced by linear DNA (38). Supercoiled DNA fails to activate DNA-PKCS, but will bind the enzyme, presumably occupying the open channel. It is possible that the three-component interaction of supercoiled DNA, C1D, and DNA-PKCS induces a conformational change in DNA-PKCS that activates the kinase domain. Unpaired single strands would activate the kinase by inducing a similar conformational change. Perhaps a specific domain of C1D interacts with DNA-PKCS in a way that mimics the effect of single-stranded DNA.

In conclusion, we have discovered that DNA-PKCS is activated by DNA via its interaction with single-stranded ends. In this case, the kinase may direct the phosphorylation of target proteins involved in DNA repair. DNA-PK might also be capable of co-localization and phosphorylation of a specific target protein, such as the glucocorticoid receptor. In this case, the effect appears to require precise juxtaposition of the target protein, because the kinase would be only minimally activated. Finally, DNA-PK might be fully activated in the absence of DNA ends by interacting with C1D. Interestingly, C1D transcription is strongly induced by ionizing radiation (38). Here, DNA-PK might phosphorylate target proteins as part of a signaling pathway triggered by ionizing radiation.

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