The single-stranded (ss) DNA-binding protein LEF-3 of *Autographa californica* multinucleocapsid nucleopolyhedrovirus promoted Mg$^{2+}$-independent unwinding of DNA duplexes and annealing of complementary DNA strands. The unwinding and annealing activities of LEF-3 appeared to act in a competitive manner and were determined by the ratio of protein to DNA. At sub-saturating and saturating concentrations, LEF-3 promoted annealing, whereas it promoted unwinding at oversaturation of DNA substrates. The LEF-3 binding to ssDNA and unwinding activity were sensitive to redox agents and were inhibited by oxidation of thiol groups in LEF-3 with 1,1′-azobis(N,N-dimethylformamide) (diamide) or by modification with the thiol-conjugating agent N-ethylmaleimide. Both oxidation and alkylation increased the dissociation constant of the interaction with model oligonucleotides indicating a decrease in an intrinsic affinity of LEF-3 for ssDNA. These results proved that free thiol groups are essential both for LEF-3 interaction with ssDNA and for DNA unwinding. In contrast, oxidation or modification of thiol groups stimulated the annealing activity of LEF-3 partially due to suppression of its unwinding activity. Treatment of LEF-3 with the reducing agent dithiothreitol inhibited annealing, indicating association of this activity with the oxidized protein. Thus, the balance between annealing and unwinding activities of LEF-3 was determined by the redox state of protein with the oxidized state favoring annealing and the reduced state favoring unwinding. An LEF-3 mutant in which the conservative cysteine Cys$^{214}$ was replaced with serine showed both a decreased binding to DNA and a reduced unwinding activity, thus indicating that this residue might participate in the regulation of LEF-3 activities.

A member of the family Baculoviridae, *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) has a circular double-stranded DNA genome of 134 kb that encodes ~150 genes (1). AcMNPV is widely used for the generation of recombinant viruses for the expression of foreign genes and as a model for analysis of baculovirus replication. Six viral factors, including a transactivator of early gene transcription IE-1, DNA polymerase, DNA helicase, a DNA primase called LEF-1 (late expression factor 1), primase-associated factor LEF-2, and a single-stranded DNA-binding protein (SSB) LEF-3 are essential for replication of viral DNA in transient assays (2, 3). AcMNPV LEF-3 has a molecular mass of 44.5 kDa and forms multimers in solution (4). It binds specifically to ssDNA (5) and promotes Mg$^{2+}$- and ATP-independent unwinding of partial DNA duplexes (6). In nuclei of infected cells, LEF-3 localizes to virus replication factories and virogenic stroma (7). The abundance of this protein in infected cells, its preferential binding to ssDNA, and its unwinding activity suggest that LEF-3 is a member of a diverse family of SSBs proteins. Despite minimal sequence homology and a highly diverse structure, different SSBs likely play similar roles in DNA metabolism. By destabilizing DNA duplexes, SSBs facilitate various transitions of DNA that accompany replication, repair, and recombination. In addition, they serve as accessory factors for other proteins and enzymes. LEF-3 has been demonstrated to interact with baculovirus DNA helicase (8–10) and alkaline nuclease (11). The accessory role of LEF-3 in functional complexes with these viral enzymes, along with its ability to alter DNA structure, suggests that it plays a central role in the virus infection cycle. It remains unclear how the multiple functions of LEF-3 are regulated in vivo, but numerous experimental data suggest possible involvement of redox factors in regulation of other DNA-binding proteins. The redox state determines interaction with DNA of the multifunctional eukaryotic SSB protein RP-A (12), the major DNA-binding protein ICP8 of herpes simplex virus type 1 (13, 14), and many transcription factors, including Fos and Jun (15), NF-κB (16), Pax (17), FNR (18), Oxy R (19) (reviewed in Ref. 20), and the E2 protein of bovine papillomavirus type 1 (21).

In this report, we describe the effect of redox agents on activities of the baculovirus SSB protein LEF-3. The DNA binding and unwinding activities of LEF-3 were strongly inhibited by modification or oxidation of free sulfhydryl groups in the protein. We found a new activity of LEF-3: the annealing of complementary DNA strands, which was stimulated by oxidation and appeared to be associated specifically with the oxidized protein. These data suggest a plausible mechanism by which redox factors may regulate the function of LEF-3.
EXPERIMENTAL PROCEDURES

Chemicals—N-Ethylmaleimide (NEM) was from Sigma, 1,1′-azo-bis(N,N′-dimethylformamide) (diamide) was from TCI America, tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP), and 4-acetamido-4-maleimidostilbene-2,2′-disulfonic acid, disodium salt (AMS) were from Molecular Probes.

Cells and Recombinant Baculoviruses—Spodoptera frugiperda 9 (SF9) cells were cultured in SF900II serum-free media (Invitrogen), penicillin G (50 units/ml), streptomycin (50 μg/ml, Whittaker Bioproducts), and fungizone (ampicillin B, 375 ng/ml, Flow Laboratories) as previously described (22). For overexpression of His-tagged LEF-3, the Bac-to-Bac baculovirus expression system (Invitrogen) was used following the manufacturer’s instructions. The pRh5-HTa-LEF3 transfer plasmid was constructed by inserting the LEF-3 open reading frame into the Ncol-NotI sites in pFastBac HTa (Invitrogen). To replace Cys with Ser at LEF-3 position 214, a point mutation was introduced in the transfer plasmid by using a Transformer site-directed mutagenesis kit (Clontech). The selection primer (ATTCGTACTGAGCTTGAGATGTGCTCAAGCTTTATGCCGTT; 62-mer, TGGGTGAACCTGCAGGTTCACCCA; and 25-mer, AACGGCATACTGAGCTTGCGAGTTTG; GGGCAAAGATGTCCTAGCAATGTAATCGTCAAGCTTTATGCCGTT; TCTAGAGGATCCGACTATCGA; 62-mer, TGGGTGAACCTGCAGGTTTCACCCA; and 25-mer, AGGCGCATAAAGCTGAGGTGTTTGCAGTTTG; and other ingredients of the standard assay mixture. Titration with LEF-3 was carried out in reactions containing 1 μM 32P-labeled 62-mer or 63-mer and other members of the standard assay plus 0.5% Nonidot P-40. The labeled double-stranded substrate, ds 62-mer, was used at a concentration of 0.1 μM. Sulfhydryl reagents, NEM or 1,1′-azo-bis(N,N′-diamide) (diamide) were added to the reaction both before and after DTT before LEF-3. The binding reactions were incubated for 15 min at 22 °C and then analyzed by electrophoretic mobility shift assay (EMSA) using a 6% polyacrylamide gel as described previously (25). The relative amount of unbound (free − f) DNA in each probe was counted, and the bound DNA (b) was determined as b = 100% − f. The unwinding and annealing reactions were incubated for 1 h at 30 °C, then treated with 20 mM Tris-HCl, pH 8.5, 0.1 M NaCl, 50 mM KCl, 1% SDS, and then loaded in different amounts on separate lanes of the same gel was used for generation of the calibration curve. For quantitative analysis, the stained gels and the fluorescence images were analyzed with ImageQuant software (Amersham Biosciences).

RESULTS

The annealing and unwinding activities of LEF-3 were assayed in reaction mixtures containing 63-mer and 62-mer oligonucleotides that were capable of forming a Y-shaped structure with a 37-bp duplex region and single-stranded (ss) 5′- and 3′-tails. The ss tails provided LEF-3 with binding sites required for unwinding the duplex region. To monitor structural transitions in the DNA substrates using PAGE and autoradiography, the 63-mer oligonucleotide was labeled with 32P. The assays were carried out in the presence of 0.5 mM 63-mer and 0.75 mM 62-mer oligonucleotides that provided a total of ~5 nM binding sites for LEF-3 assuming its binding site size is ~10 nt (5, 6). For the annealing assay, the oligonucleotides were mixed directly before incubation with LEF-3, whereas for the unwinding assay, the oligonucleotides were pre-annealed before adding LEF-3. The dose dependence for the annealing and unwinding reactions in the presence of reducing reagent (2 mM and 20 mM DTT) and oxidizing reagent (20 mM diamide) are shown in Fig. 1. In the presence of 2 mM DTT, LEF-3 promoted the annealing of the oligonucleotides with the concentration optimum near 10 nm that approximately corresponds to saturation of the DNA by LEF-3. Under the same conditions, LEF-3 promoted unwinding of preformed Y-shaped structures at concentrations higher than 30 nM, i.e. at oversaturation of DNA substrates. The increase in DTT concentration to 20 mM suppressed the annealing (Fig. 1A) but elevated the unwinding activity (Fig. 1B and C). Diamide, which oxidizes sulfhydryl groups in cysteines (27), produced the opposite effect, an efficient inhibition of unwinding, and a marked stimulation of
The annealing and unwinding assays were carried out in standard 10-K (150 mM) solution when diamide (5 mM) was added together with excess of the reducing agent DTT (2 or 20 mM) and diamide (10 mM) were added as indicated. The 60-μl reaction mixtures were assembled on ice then transferred to incubation at 50 °C, and 10-μl aliquots were taken at different time points during 90-min incubation. The samples were treated with SDS and proteinase K and then analyzed by PAGE. The filled symbols represent the respective reactions after incubation for 90 min at 30 °C in the absence of LEF-3.

The amount of ds 63-mers in the annealing reaction (1.8%) and the amount of ss 63-mers in the unwinding reaction (7.8%) at time zero was subtracted from the values obtained.

![Figure 1](image.png)

**Figure 1.** Annealing of DNA strands and unwinding of partial DNA duplexes by LEF-3 at different redox conditions. A and B, the annealing and unwinding assays were carried out in standard 10-μl reaction mixtures containing 0.5 mM 32P-labeled 63-mer, 0.75 mM 62-mer, 40 mM Tris-HCl, pH 7.5, 50 mM NaCl, 12.5% glycerol, and 100 μg/ml BSA. For the unwinding assay, the 63-mer and 62-mer oligonucleotides were pre-annealed before the reaction. The reactions contained DTT or diamide as indicated. LEF-3 was added in the following concentrations: control, no LEF-3 (lane 1), 5.6 nM (lane 2), 11.3 nM (lane 3), 22.5 nM (lane 4), 45 nM (lane 5), 90 nM (lane 6), 180 nM (lane 7), 280 nM (lane 8), 450 nM (lane 9), and 900 nM (lane 10). The reactions were incubated for 1 h at 30 °C, then treated with SDS (0.5%) and proteinase K (150 μg/ml) for 15 min at 22 °C, and analyzed by PAGE. Diagrams of the DNA substrates are shown at the right. The asterisk indicates the radioactive label in the 63-mer. C, the relative amount of the labeled 63-mer that was present in a duplex with the 62-mer in the annealing reactions shown in panel A (open symbols), and the relative amount of the labeled 63-mer released from the duplex in the unwinding reactions shown in panel B (filled symbols).

The stoichiometry of the reaction, whereas later the unwinding proceeded at similar rates in the presence of 20 and 2 mM DTT (Fig. 2B). As shown previously, monovalent and divalent salts (100 mM NaCl or 5–10 mM MgCl2) are inhibitory for the unwinding activity of LEF-3 (6). In this report, the annealing and unwinding reactions were carried out in mixtures containing 50 mM NaCl and no divalent cations. The addition of MgCl2 in concentrations of 1 or 2 mM did not substantially change the annealing activity of LEF-3. Further increase in MgCl2 concentration to 5 mM decreased the annealing and shifted the LEF-3 optimum from 10 to 40 mM, but did not eliminate the stimulatory effect of diamide. The shift in LEF-3 optimum to a higher protein concentration was also observed with an increase in NaCl concentration to 100 and 150 mM (data not shown). The higher protein amounts are required for annealing presumably due to a less efficient protein interaction with DNA strands in the presence of salts. Analysis of LEF-3 binding to ssDNA by using EMSA showed that NaCl concentrations higher than 50 mM decrease LEF-3 affinity for ssDNA (data not shown). Although the Mg- and ATP-independent DNA unwinding activity of baculovirus LEF-3 was previously described (6, 28), the capacity of LEF-3 to promote the annealing of complementary DNA strands is demonstrated here for the first time. The data shown in Fig. 1 allow predictions about the interplay of both LEF-3 activities: (i) LEF-3 promotes the annealing of complementary strands or the unwinding of DNA duplexes depending on a protein to DNA ratio: at concentrations near or below the saturation level, LEF-3 facilitates annealing, whereas it promotes unwinding at oversaturation; (ii) the LEF-3 activities depend on the redox state of the protein; free sulfhydryl groups are essential for unwinding, but not for annealing activity. The latter prediction suggests that LEF-3 action in the course of DNA unwinding differs structurally from its action upon DNA annealing. The next experiments were designed to test these predictions.
FIG. 3. Inhibition of the unwinding activity by alkylation or oxidation of sulfhydryl groups in LEF-3. The unwinding assay was carried out in 10-μl reaction mixtures containing 0.5 nM 32P-labeled 25-mer pre-annealed to 1 nM 62-mer. The sulfhydryl reagents DTT, NEM, and diamide were added as indicated. After addition of LEF-3 (280 nM), the reactions were incubated for 1 h at 30 °C, and then treated by SDS and proteinase K and analyzed by PAGE. In each experiment, the amount of 25-mers released from the duplex in the absence of sulfhydryl reagents was taken as 100%. The other bars represent the average relative amount (from three experiments) with standard deviation of 25-mers released in the presence of sulfhydryl reagents. The sequence of the partial DNA duplex used in the assay is shown below the panel.

The inhibition of the unwinding activity of LEF-3 by chemicals that react with thiol groups in cysteines was observed in experiments with partial DNA duplexes having different sequences and secondary structures. The effect of DTT, NEM, and diamide on the unwinding of the 25-mer duplex having a 37-mer 5′-tail is shown in Fig. 3. The addition of the reducing agent DTT markedly stimulated the unwinding of the partial duplex, whereas the oxidation of thiol groups in LEF-3 by diamide or their covalent modification by the thiol-conjugating agent NEM efficiently inhibited the unwinding. These data are in agreement with the prediction that free sulfhydryl groups in cysteines are essential for the unwinding activity of LEF-3.

Although, the oxidizing agent diamide inhibited DNA unwinding, it markedly stimulated the annealing activity of LEF-3 (Figs. 1 and 2). In addition, it shifted the LEF-3 optimum for annealing to higher protein concentrations. Due to this shift, diamide produced different quantitative effects at different protein concentrations. It caused only a moderate increase (~2-fold) in annealing in a protein range of 5–11 nM, but it produced a much higher stimulation at protein concentrations in a range of 22–45 nM (Figs. 1 and 4A). Alkylation of sulfhydryl groups in LEF-3 by NEM also resulted in a more pronounced increase in annealing at the higher protein concentrations, although the overall stimulation was lower than that for diamide (Fig. 4A). In the presence of 5 mM NEM, LEF-3 promoted the annealing at a rate higher than that at 2 mM DTT, but lower than that at 10 mM diamide over the 90-min incubation period at standard conditions (data not shown). The stimulation of annealing and the shift in the LEF-3 optimum with treatment by NEM and diamide might be intrinsically connected to the inhibition of the unwinding activity of LEF-3 by these reagents. The annealing and unwinding activities promote structural transitions in DNA substrates in opposite directions, and these activities of LEF-3 appeared to act in a competitive manner. The oversaturating amounts of LEF-3 not only stimulated unwinding but also inhibited annealing. At the standard assay temperature of 30 °C, 12–14% of 63-mers formed the Y-shaped partial duplexes with 62-mers in the absence of LEF-3, whereas the yield of the duplexes dropped to 4–6% at LEF-3 concentrations higher than 90 nM (Fig. 1C). This was further confirmed by an experiment in which the annealing assay was carried out at 37 °C, when ~25% of 32P-labeled 63-mers were annealed with 62-mers in the absence of LEF-3 (Fig. 4B). Addition of LEF-3 in subsaturating and saturating amounts (5 and 11 nM) stimulated the generation of duplexes, but the accumulation of duplexes was inhibited at LEF-3 concentrations higher than 50 nM, when the yield of nascent duplexes dropped to 3% or less. This result suggested that at concentrations above the saturation level, when LEF-3 promotes unwinding of the preformed DNA duplexes, it also inhibits accumulation of nascent duplexes that resulted from annealing of complementary strands during incubation. There-
fore, unwinding may mask the annealing activity of LEF-3. By selective inhibition of the unwinding activity of LEF-3, diamide and NEM might increase the yield of nascent duplexes in the annealing assay. Because unwinding required higher doses of LEF-3 than annealing, the inhibition of unwinding serves to shift the optimum for annealing to higher protein concentrations as was observed in experiments shown in Figs. 1 and 4A.

Both NEM and diamide react with thiol groups in cysteines, but the alkylating agent NEM produces stable adducts with cysteines and blocks them, whereas diamide oxidizes free sulfhydryl groups to sulfenic acid that may react to form intra- or inter-protein disulfide bonds. Both NEM and diamide inactivated the DNA-unwinding capacity of LEF-3 (Figs. 1–4). However, diamide produced much stronger stimulatory effects on the unwinding capacity of LEF-3 than annealing, the inhibition of unwinding serves to shift the optimum for annealing to higher protein concentrations as was observed in experiments shown in Figs. 1 and 4A.

The standard 10-μl reactions contained 0.2 nM 32P-labeled linear 70-mer (lanes 1–4) or minicircle 70-mer (lanes 5–8). DTT (lanes 3 and 7) and NEM (lanes 4 and 8) were each added to a final concentration of 5 mM. After addition of LEF-3 (27 nM), the reactions were incubated for 20 min at 22 °C, and then analyzed by EMSA. Lane 1 and 5 represent control reactions lacking LEF-3. B, the effect of diamide on LEF-3 binding to 70-mer DNA. The standard 10-μl reactions contained 0.4 nM 32P-labeled linear 70-mer and other ingredients except DTT. Diamide was added to 1 mM (lanes 3 and 5) or 5 mM (lanes 4 and 6), and DTT to 50 mM (lane 7). After addition of LEF-3 (36 nM), the mixtures were incubated for 15 min at 22 °C. The reactions shown in lanes 1 through 4, and 7 were directly loaded onto a 6% polyacrylamide gel and analyzed by EMSA, whereas DTT (20 mM) was added to the reactions shown in lanes 5 and 6, and the incubation continued for 15 min before EMSA. Lane 1 represents a control reaction lacking LEF-3. C and D, titration of 32P-labeled ss 62-mer and ds 62-mer with LEF-3 by using EMSA. The binding reactions were carried out in 10-μl mixtures containing 1 nM of 32P-labeled ss 62-mer (open symbols) or 0.1 nM of 32P-labeled ds 62-mer (filled symbols), 30 mM Tris-HCl, pH 7.5, 50 mM NaCl, 12.5% glycerol, 100 μg/ml BSA, and 0.5% Nonidet P-40. The sulfhydryl reagents 5 mM DTT, 5 mM NEM, and 10 mM diamide were added as indicated. After addition of LEF-3, the reactions were incubated for 15 min at 22 °C, and then analyzed by EMSA. D, analysis of LEF-3 binding to ss 62-mer (lanes 1–5) and to ds 62-mer (lanes 9 and 10). LEF-3 was added in the following concentrations: control, no LEF-3 (lanes 3 and 9), 2.3 nM (lane 1), 4.5 nM (lane 2), 9 nM (lane 3), 18 nM (lane 4), 36 nM (lane 5), 72 nM (lane 6), 144 nM (lane 7), and 210 nM (lane 10). The position of the complexes with the predicted LEF-3 monomer, dimer, trimer, and high molecular weight oligomer are marked, respectively, “+1,” “+2,” “+3,” and by an asterisk on the right.

It was unclear which step in the unwinding reaction requires free thiols. By using EMSA, we demonstrated that oxidation or alkylation of LEF-3 thiols decreased the protein affinity for ssDNA (Fig. 5). During incubation with labeled 70-mer oligonucleotide, LEF-3 formed a complex that migrated slower than unbound 70-mer (Fig. 5A). Incubation in the presence of the reducing agent DTT markedly increased the yield of stable complexes (lane 3), whereas the thiol-conjugating agent NEM prevented generation of complexes (lane 4). To avoid possible interference of the 5′ and 3′-tails of the 70-mer oligonucleotide with LEF-3 binding, we repeated the binding experiment with a minicircle DNA that was prepared from the linear 70-mer (lanes 5–8). The effect of both redox agents on LEF-3 binding to the circular 70-mer was the same as with the linear 70-mer. LEF-3 binding was stimulated by DTT (lane 7), but it was inhibited by NEM (lane 8). In further experiments, we found that the oxidizing agent diamide decreased the yield of LEF-3 complexes with the 70-mer probe (Fig. 5B, lanes 3 and 4). As expected, incubation of the LEF-3 samples oxidized by diamide with DTT in excess restored protein binding to DNA (compare lanes 5 and 6 to lanes 3 and 4 in Fig. 5B). Thus, diamide and DTT can reversibly change the affinity of LEF-3 for DNA by oxidation or reduction. The data indicated that LEF-3 with reduced thiols can form a more stable complex with ssDNA than protein with oxidized or modified thiols. To estimate quantitatively the LEF-3 interaction with DNA at different redox conditions, we titrated a 62-mer oligonucleotide with
LEF-3 in the presence of DTT, diamide, and NEM and determined the yield of LEF-3 complexes with the DNA probe by using EMSA (Fig. 5C). To minimize nonspecific protein-protein interaction, 0.5% Nonidet P-40 was added to the binding mixtures in all titration experiments. In the presence of 5 mM DTT, the half-saturation of 1 nM ss 62-mer was observed at a LEF-3 concentration of 12 nM. Oxidation of LEF-3 with diamide or alkylation with NEM markedly reduced the LEF-3 binding. The half-saturation of the ss 62-mer in the presence of 10 mM diamide and 5 mM NEM was observed at LEF-3 concentrations of 21 and 31 nM, respectively. The increase in the annealing capacity of LEF-3 after oxidation with diamide (Figs. 1 and 4) might have resulted from an elevated affinity of the oxidized protein for double-stranded DNA. To determine if this occurred, we compared LEF-3 binding to a ds 62-mer in the presence of DTT and diamide. The half-saturation of 0.1 nM ds 62-mer in the presence of 5 mM DTT and 10 mM diamide was at LEF-3 concentrations of 0.32 and 0.48 μM, respectively. This result confirmed the specific affinity of LEF-3 for ssDNA and also demonstrated that the binding to dsDNA is inhibited by diamide in the same manner as the binding to ssDNA. The retardation pattern under EMSA (Fig. 5D) indicated that LEF-3 interacts with the ss 62-mer as a protein oligomer, presumably a trimer, which presents a predominant form of LEF-3 in solution (4). At low concentrations, LEF-3 formed one shifted band with the ss 62-mer (lane 1), which showed gradually decreasing mobility as the LEF-3 concentration was increased (lanes 2–7). A different pattern was observed in the experiment with the ds 62-mer, which has a slightly higher mobility in the gel than the ss 62-mer (compare lanes 9 and 10). The complexes with the ds 62-mer might contain LEF-3 monomers and dimers (marked, respectively, by “×1” and “×2” in lane 10) in addition to higher protein oligomers (marked by “×3” and asterisk). Because the 62-mer oligonucleotide is long enough to provide several binding sites for LEF-3, the interaction with this probe depends on both the intrinsic LEF-3 affinity for DNA and the cooperativity of binding. Although LEF-3 appeared to bind the ds 62-mer with lower cooperativity than the ss 62-mer, the retardation patterns with both DNA probes revealed intensive protein-protein interactions that accompanied LEF-3 binding to DNA. To clarify how the redox state affects the intrinsic affinity of LEF-3 for ssDNA, we repeated the titration experiments with oligonucleotides dTn that provide only one binding site for LEF-3. Under cooperative binding, LEF-3 saturates long ssDNA at a ratio of one protein monomer to ~10 nt of DNA (5, 6). LEF-3 indeed caused retardation of dT12 under the standard analysis by EMSA, but its affinity for dT12 was much lower than that for dT17 (Fig. 6B). The half-saturation of 1 nM dT12, dT17, dT24, and dT34 was observed at LEF-3 concentrations of 96, 23, 20, and 17 nM, respectively. The mobility of the major LEF-3/dTn complex in the polyacrylamide gel corresponded to the binding of a LEF-3 oligomer, presumably a trimer. Titration of dT17 with increasing concentrations of LEF-3 caused the major band to split into two adjacent bands (Fig. 6A, lanes 7 and 9). This result suggested that each LEF-3 oligomer has two available DNA-binding sites. At low LEF-3 concentration, both sites were occupied by dT17 that was present in excess, and only one primary band with low mobility was seen (lanes 3 and 5). Under increasing LEF-3 concentrations, a second band with higher mobility was evident. It likely corresponds to a LEF-3 oligomer with only one site occupied by dT17. This band became predominant at higher concentrations (lane 9). This pattern was minimal with dT34 (lanes 4, 6, 8, and 10). Thus, the oligonucleotide dT34 was able to occupy both DNA-binding sites of the LEF-3 oligomer, or the LEF-3 oligomer has only a single DNA-binding site, but it is large enough to provide room for two dT17 oligonucleotides. In the next experiment, LEF-3 was treated with 10 mM diamide or 5 mM NEM for 30 min on ice, and then it was analyzed for binding to dT17 (Fig. 6, C and D).
Both treatments, oxidation and alkylation, markedly decreased the affinity of LEF-3 for dT17. The half-saturation of 1 mM dT17 was observed at LEF-3 concentrations of 120 and 110 nM for samples treated, respectively, by diamide and NEM, whereas the control LEF-3 sample showed half-saturation at 23 nM. The decrease in affinity for ssDNA was observed after preincubation of LEF-3 with diamide before the binding reaction or after its addition into the mixture containing both LEF-3 and dT17. When 10 mM diamide was added directly to the binding reaction, the half-saturation of dT17 was observed at 92 nM LEF-3. Similar data were obtained in the experiment with dT34. The half-saturation of 1 mM dT34 was observed at LEF-3 concentrations of 94 and 59 nM for the samples pretreated, respectively, with 10 mM diamide and 5 mM NEM, whereas it was observed at 17 nM LEF-3 at the reducing condition (5 mM DTT). Assuming that LEF-3 binds dT34 as a trimer with one binding site, we calculated the dissociation constants ($K_d$) as the concentration of free protein at half-saturation. The $K_d$ values for interaction of LEF-3 with dT34 at the reducing conditions and after the treatment by diamide and NEM were equal to 0.51 for binding of two LEF-3 trimers, the pattern shown in Fig. 6. The retardation pattern in polyacrylamide gels revealed that oxidation and alkylation of free thiols in LEF-3 actually decreased the intrinsic affinity of this protein for ssDNA. The retardation pattern in polyacrylamide gels revealed that the treatment by diamide increased the content of high molecular weight oligomers in LEF-3 samples. The faint band of high molecular weight oligomers, which is barely seen at the reducing conditions (marked by an asterisk in Fig. 6A, lane 3) became predominant in the binding reaction with the oxidized LEF-3 (marked by an asterisk in Fig. 6C). This band was the one seen previously in the experiment with the ds 62-mer (Fig. 5D, lane 10), and it reflected presumably binding of two LEF-3 trimers. Because dT17 did not provide a site for binding of two LEF-3 trimers, the pattern shown in Fig. 6C suggested dimerization of LEF-3 trimers under oxidation. The high molecular weight LEF-3 oligomer appeared to be a poor competitor to the regular LEF-3 trimer in binding to dT17, because the band of LEF-3 high molecular weight oligomers disappeared as the concentration of reduced LEF-3 increased (see asterisk, Fig. 6A). This result suggested that oligomerization of oxidized LEF-3 might cause a decrease in the affinity of the protein for ssDNA.

The sensitivity of the DNA binding, annealing, and unwinding activities of LEF-3 to redox reagents suggested an essential role for cysteine thiols in regulating LEF-3 function. AcMNPV LEF-3 has eight cysteines. To clarify the redox state of these cysteines in LEF-3 samples, we used non-reducing SDS-PAGE (Fig. 7). The omission of reducing reagents (DTT or 2-mercaptoethanol) from protein samples before SDS-PAGE allows the detection of species that are cross-linked by inter- and intra-disulfide bonds. The intra-cross-linked LEF-3 species migrated as a diffuse band faster than the reduced monomer, whereas the inter-cross-linked species were present predominantly as dimers (lane 2). The addition of 20 mM DTT to LEF-3 sample before denaturation and SDS-PAGE eliminated the cross-linked species and resulted in a pattern typical for reducing conditions of electrophoresis (lane 1). The addition of cysteine generated predominantly species migrating slightly more slowly than the reduced monomer (compare lanes 3 and 1) presumably due to reaction of the added cysteines with disulfides in LEF-3, and it also eliminated most of intra- and inter-cross-linked species. Preincubation of LEF-3 with 10 mM diamide for 1 h at room temperature before SDS-PAGE markedly increased the yield of cross-linked oligomers (lane 4), thus indicating that oxidation by diamide stimulates LEF-3 oligomerization. Depending on the concentration, the addition of DTT to the oxidized samples suppressed or completely reversed the diamide effect (lanes 5-7). The possible presence of disulfide bonds in LEF-3 was assayed by covalent modification of free sulphydryl groups by the thiol-conjugating agents NEM (molecular mass = 125 Da) and AMS (molecular mass = 536 Da). If all eight thiols in LEF-3 are reduced and can react with these agents, alkylation by NEM and AMS should increase the molecular mass of LEF-3 by 1 and 4.3 kDa, respectively. The disulfide bonds in LEF-3 would be revealed by presence of the cross-linked species. The LEF-3 samples were incubated in the presence of 10 mM NEM or 10 mM AMS for 30 min at room temperature and then subjected to non-reducing SDS-PAGE (lanes 8 and 9). Most of the protein in each sample treated with these alkylators migrated as one shifted band with a mobility that was consistent with alkylation of all cysteines in LEF-3. However, a minor fraction in each sample was present as intra- and inter-cross-linked species. Disulfide bonds in these species might have been generated during processing of the samples and SDS-PAGE if some thiols in LEF-3 did not react or reacted slowly with the alkylators. Another possibility is that a minor fraction in the LEF-3 samples was oxidized and contained disulfide bonds prior to the analysis. To reduce the oxidized cysteines, the LEF-3 samples were preincubated with the reducing agent TCEP prior to alkylation. When the LEF-3 samples were preincubated for 15 min with 5 mM TCEP and then treated by NEM or AMS, the alkylated protein migrated as one uniform band and did not show the presence of cross-linked species (lanes 10 and 11). These data suggested that LEF-3 does not contain disulfide bonds as a regular structural element, although free thiols in LEF-3 samples might be oxidized and then generate species cross-linked by disulfides.

AcMNPV LEF-3 has eight cysteines, but it does not contain motifs that are involved in the redox regulation of other known DNA-binding proteins. Only one cysteine, Cys214, is conserved in baculoviral homologs of LEF-3. To elucidate the role of this cysteine residue in LEF-3 function, we constructed a LEF-3 mutant with the replacement of cysteine with serine at position 214. We expected that a mutant LEF-3 lacking an essential cysteine might reveal a deficiency in DNA binding and unwinding. The LEF-3(C214S) mutant and wild type (wt) proteins...
Redox Regulation of the Baculovirus SSB Protein LEF-3

were expressed in recombinant baculoviruses as N-terminal His<sub>6</sub> tag fusions. The His-tagged mutant and wt proteins were purified free of wild type non-tagged LEF-3 by using chromatography on nickel-nitrilotriacetic acid columns. Due to possible differences in affinity of the wt and mutant LEF-3 for ssDNA, chromatography on a ssDNA-column was excluded from the standard protocol for LEF-3 purification (see “Experimental Procedures”). Under SDS-PAGE, the purified proteins showed similar patterns when the samples were processed in the presence of the reducing agent DTT (Fig. 8, lanes 1 and 2). However, when processed using oxidizing conditions, the wt and mutant LEF-3 generated unique sets of intra- and inter-cross-linked species (lanes 3 and 4). These data confirmed the involvement of Cys<sup>214</sup> in the production of cross-linked species when LEF-3 is oxidized and denatured. Partial trypsin digestion of the wt and mutant LEF-3 species produced the same set of resistant fragments thus indicating that the global domain structure of LEF-3(C214S) was similar to wt LEF-3 (data not shown). Analysis by EMSA demonstrated that LEF-3(C214S) was able to form a stable complex with the 63-mer oligonucleotide probe, although the mutant revealed a lower affinity for DNA than wt LEF-3 (Fig. 9). The half-saturation of 1 nm 63-mer was observed at protein concentrations of 13 and 22 nM for the wt and mutant LEF-3, respectively. However, LEF-3(C214S) binding to the oligonucleotide probes remained sensitive to redox agents, as shown above for wt LEF-3 (see Fig. 5A). The binding was stimulated by the reducing agent DTT and blocked by the alkylator NEM (data not shown). The unwinding and annealing activity of LEF-3(C214S) was also examined. The mutant LEF-3 had ~2- to 2.5-fold lower specific unwinding activity than wt LEF-3. It possessed 44 ± 5% the activity of wt LEF-3, when it was analyzed at three oversaturating protein concentrations of 130, 250, and 450 mM using the standard unwinding assay employing the Y-shaped partial duplex shown in Fig. 1. In contrast, LEF-3(C214S) showed ~1.5- to 2-fold higher (170 ± 30%) annealing activity than wt LEF-3. This activity was determined in two independent experiments, at two protein concentrations near the optimum (5.6 and 11 mM) by using the standard annealing assay described in Fig. 1. These data suggest that Cys<sup>214</sup> likely plays a functional role in LEF-3, but that other LEF-3 cysteines also contribute to its sensitivity to redox agents. We also characterized four additional LEF-3 mutants each with replacement of single cysteines with serines at positions 82, 171, 238, and 252 that were considered most promising for regulating LEF-3 activity. The three residues, Cys<sup>82</sup>, Cys<sup>238</sup>, and Cys<sup>252</sup>, are inside the putative DNA-binding sites of LEF-3, which are located at positions from Lys<sup>34</sup> to Lys<sup>104</sup> and from Lys<sup>183</sup> to Lys<sup>236</sup>. In AcMNPV LEF-3, Cys<sup>82</sup> is located between two amino acids, Lys<sup>81</sup> and Tyr<sup>83</sup>, which may potentially directly interact with DNA. In addition, Tyr<sup>83</sup> is well conserved in LEF-3 proteins. The other three LEF-3 cysteines, Cys<sup>305</sup>, Cys<sup>336</sup>, and Cys<sup>356</sup>, are located apart from putative DNA-binding motifs, and they were not analyzed. Each LEF-3 mutant was expressed in a recombinant baculovirus as a N-terminal His<sub>6</sub> tag fusion and analyzed by SDS-PAGE, and by using the DNA binding, annealing, and unwinding assay as described above for the mutant C214S. Under non-reducing SDS-PAGE, each mutant protein generated a specific set of intra- and inter-cross-linked species that differed from those formed by wt LEF-3 (data not shown). This result confirmed a capacity of each of the four cysteines to form disulfide bonds with other cysteines. The mutant with the replacement of Cys<sup>82</sup> had ~2- to 2.5-fold lower specific unwinding activity than wt LEF-3, whereas all mutants except one (C252S) had enhanced annealing activity. However, the DNA-binding activity of all the mutant LEF-3 species was sensitive to redox reagents and was stimulated by reducing agent DTT and blocked by alkylator NEM, as shown above for wt LEF-3 (see Fig. 5A).

LEF-3 is essential for virus production. When we deleted the lef-3 gene from the AcMNPV bacmid, no viable virus was detected after transfection into Sf9 cells (data not shown). To elucidate a possible role of LEF-3 Cys<sup>214</sup> in the baculovirus infection cycle, we constructed two viruses with the lef-3 gene replaced by a construct encoding His-tagged wt LEF-3 or mutant LEF-3(C214S) as described under “Experimental Procedures.” Sf9 cells were infected with these viruses, and the production of viable virus was analyzed at 24, 48, and 96 h post-infection. Although the mutant LEF-3 showed altered biochemical properties, the virus expressing the mutated lef-3 gene produced infectious viruses at the same level as the control virus expressing His-tagged wt LEF-3 (data not shown). These results indicate that the LEF-3(C214S) mutant with the decreased DNA-binding and unwinding efficiency is capable of supporting virus replication as well as wt LEF-3 under the assay conditions tested. Four other bacmids expressing mutant LEF-3 species with replacement of cysteine with serine at positions 82, 171, 238, and 252 also produced infectious virus at a level close to a control bacmid expressing His-tagged wt LEF-3 (data not shown).

**DISCUSSION**

The DNA binding, unwinding, and annealing activities of SSB proteins are thought to play a crucial role in diverse structural transitions of DNA during replication, repair, and recombination. The activities of the baculovirus SSB protein
LEF-3 showed a high sensitivity to redox reagents in experiments in vitro with different DNA substrates (Figs. 1–6). DNA binding and unwinding were inhibited with the oxidizing agent diamide and the thiol-conjugating agent NEM, thus indicating that sulfhydryl groups of cysteines are essential for these LEF-3 activities. The experiments with oligonucleotides dT₆₆ proved that oxidation and alklylation of LEF-3 cysteines decreased the intrinsic affinity of this protein for ssDNA. It remains unclear whether LEF-3 cysteines are directly involved in interaction with DNA or they are required for structural transitions that generally accompany the binding of SSB proteins to DNA (29–34). In contrast to unwinding, DNA annealing was stimulated by oxidation or alklylation of thiol groups in LEF-3. Due to competition of the annealing and unwinding activities of LEF-3, the annealing stimulation might result, at least partially, from the inhibition of unwinding. On the other hand, the high level of stimulation by diamide and the inhibition of the annealing activity by reduction with DTT indicated specific involvement of an oxidized fraction of LEF-3 in DNA annealing. It remains to be determined how oxidation of LEF-3 enhances its annealing activity. It is possible that the oxidized LEF-3 cysteines may generate inter-protein disulfide bonds, thus stimulating production of oligomeric protein species similar to those that were previously observed in LEF-3 samples (4). The binding experiments clearly indicated the generation of LEF-3 high molecular weight oligomers presumably via dimerization of regular LEF-3 trimers under oxidizing conditions (Fig. 6C). The oligomeric species may promote annealing of complementary DNA strands by providing multiple DNA-binding sites and bringing into close proximity the interacting strands. Non-reducing SDS-PAGE showed that the LEF-3 samples we routinely prepare do not contain disulfide bonds as a regular structural element, but this analysis also confirmed that disulfides and oligomeric species may be generated by oxidation of LEF-3 thiols (Fig. 7). The presence of oxidized LEF-3 in cells infected with baculovirus has not been analyzed, therefore the physiological importance of this fraction is not known. However, baculoviruses induce oxidative stress in infected cells (35) and encode superoxide dismutase (36) presumably to diminish the consequences of this stress on the viral replication machinery. Although the purified LEF-3 samples contained only a small fraction of oxidized protein (Fig. 7), the oxidized protein may be lost under standard chromatography on ssDNA columns due to its low affinity for ssDNA. It has been shown that for another large DNA virus, herpes simplex virus-1, that oxidation of the major DNA-binding protein ICP8 may occur in vivo (13), the DNA binding of ICP8 is inhibited by NEM (37), and modification of a specific cysteine in ICP8 affects the cooperativity of its binding to DNA and its unwinding activity (14). ICP8 promotes Mg²⁺-dependent renaturation of complementary DNA strands (38) and facilitates strand exchange reactions (39–42), although this activity was not connected to the oxidized protein. Instead, the balance between annealing and unwinding activity of ICP8 can be regulated by changing the ionic conditions. At conditions for maximal unwinding (no MgCl₂ or NaCl), ICP8 promotes almost undetectable annealing, and little unwinding is seen under conditions optimal for annealing (6 mM MgCl₂, 50 mM NaCl) (38). The baculovirus SSB protein LEF-3 has little or no homology and is approximately one-third the size of the herpes simplex virus-1 protein ICP8. In contrast to ICP8, AcMNPV LEF-3 promotes Mg²⁺-independent DNA annealing and unwinding at the same ionic conditions, and the balance between both activities was determined by the protein concentration and its redox state. Although AcMNPV LEF-3 contains eight cysteines each of which may potentially serve as a target for oxidation and reduction, it does not contain zinc finger motifs, recognition sites for redox factors such as Ref-1 and thioredoxin, or motifs that are recognized by redox factors in Fos, Jun, and BPV-1 protein E2 (reviewed in Refs. 20 and 43). The Cys²¹⁴ is the only conserved cysteine in baculovirus LEF-3. It presumably plays a functional role in LEF-3, because the LEF-3 mutant with a change (C214S) had a lower affinity for ssDNA than the wt LEF-3 when analyzed by EMSA (Fig. 9) and showed approximately a 2- to 2.5-fold lower specific unwinding activity than wt LEF-3. However, DNA binding of LEF-3(C214S) was sensitive to redox agents in the same manner as the binding of wt LEF-3, thus indicating that other cysteines also contribute to the redox sensitivity of LEF-3. Four other LEF-3 mutants with replacement of single cysteines with serines at positions 82, 171, 238, and 252 showed similar redox sensitivity of DNA binding as the mutant C214S and wt LEF-3. The viruses expressing the mutant LEF-3 species were able to propagate in infected cells as well as the control virus expressing wt LEF-3. This result suggests that the low DNA binding and unwinding efficiency did not abolish LEF-3 function in vivo. However, we cannot exclude that LEF-3 abundance in infected cells might compensate for its decreased activities. It should also be noted that there is a second SSB protein encoded by many baculoviruses that could compensate for deficiencies of LEF-3. This protein, called DNA-binding protein is expressed at T-fold the level of LEF-3 and is also capable of unwinding DNA (44). Although the physiological importance of regulation of LEF-3 activities by redox factors has yet to be confirmed, data from other systems strongly support the idea that redox regulation plays a role in functioning not only of transcriptional factors (reviewed in Refs. 20 and 45), but of some SSB proteins, including eukaryotic protein RP-A (12) and viral protein ICP8 (13, 14, 37). As far as we know, the differential effect of redox agents on annealing and unwinding activities of SSB proteins was described here for the first time.

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