Identification, Cloning, and Characterization of the Bacteriophage N4 Gene Encoding the Single-stranded DNA-binding Protein

A PROTEIN REQUIRED FOR PHAGE REPLICATION, RECOMBINATION, AND LATE TRANSCRIPTION*

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Mieyoung Choi‡§, Alita Miller†, Nam-Young Cho **, and Lucia B. Rothman-Denes ‡‡

From the Departments of Molecular Genetics and Cell Biology and Biochemistry and Molecular Biology and the Department of Developmental Biology, The University of Chicago, Chicago, Illinois 60637

The coliphage N4-coded single-stranded DNA-binding protein (N4SSB) is essential for phage replication and for expression of the phage late genes, which are transcribed by the Escherichia coli σ70 RNA polymerase. As a first step in investigating the role of N4SSB in replication and transcriptional activation, we have identified and sequenced the N4SSB gene. The gene encodes a 265-amino acid protein with no apparent sequence homology to other single-stranded DNA-binding proteins. We present data indicating that N4SSB is also essential for phage recombination. Mutational analysis of the carboxyl terminus of the protein indicates that this region is required for protein-protein interactions with the N4 replication, N4 recombination, and E. coli transcriptional machineries, while the rest of the protein contains the determinants for single-stranded DNA binding.

Proteins that bind nonspecifically to single-stranded DNA with high affinity have been purified and characterized from several sources (1, 2). Single-stranded DNA-binding proteins are present in high concentration in vivo and are essential components in a variety of DNA metabolism processes. They are required for DNA replication and are also involved in repair and recombination (1). They bind to single-stranded DNA stoichiometrically and, in most cases, with positive cooperativity (3).

The N4-coded single-stranded DNA-binding protein (N4SSB) was originally detected as an activity capable of transcribing the phage late genes, which are transcribed by the Escherichia coli σ70 RNA polymerase. As a first step in investigating the role of N4SSB in replication and transcriptional activation, we have identified and sequenced the N4SSB gene. The gene encodes a 265-amino acid protein with no apparent sequence homology to other single-stranded DNA-binding proteins. We present data indicating that N4SSB is also essential for phage recombination. Mutational analysis of the carboxyl terminus of the protein indicates that this region is required for protein-protein interactions with the N4 replication, N4 recombination, and E. coli transcriptional machineries, while the rest of the protein contains the determinants for single-stranded DNA binding.

Materials and Methods

Bacterial Strains and Phages—E. coli strains W3350 (F−, thi, CouR, gal, lac) and W3350upF were used for N4 and N4am7 infection, respectively. Wild-type and amber N4 mutants were grown as described previously (7). E. coli W3350pcnB (8), provided by Dr. D. Kiino, was used as the host for the construction of the N4SSB expression plasmid. E. coli W3350pcnB(DE3), constructed by transduction of E. coli W3350(DE3) (1) with pC195 (9) to tet-resistant and in the N4SSB gene, for N4 DNA replication and late transcription. Additionally, we have found that N4SSB is essential for N4 DNA recombination. Analysis of the different phenotypes of carboxyl-terminal deletion mutants indicates that determinants of protein-protein interactions reside in a short, basic, carboxyl-terminal domain, while the rest of the protein contains the determinants of single-stranded DNA binding. Moreover, the phenotype of certain mutations indicates that the different functions of N4SSB are carried out by separate determinants.

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** Present address: Dept. of Microbiology, Daedong University, Daedong 300-716, Korea.

† † To whom correspondence should be addressed. Fax: 312-702-3172; E-mail: lbrd@midway.uchicago.edu.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) N4 U29728.

†† To whom correspondence should be addressed. Fax: 312-702-3172; E-mail: lbrd@midway.uchicago.edu.

‡‡ To whom correspondence should be addressed. Fax: 312-702-3172; E-mail: lbrd@midway.uchicago.edu.

1 The abbreviations used are: N4SSB, N4-coded single-stranded DNA-binding protein; PCR, polymerase chain reaction; ORF, open reading frame; IPTG, isopropyl-1-thio-β-D-galactopyranoside; kb, kilobases.
1.5 mM MgCl₂, 0.01% gelatin, 0.2 mM dNTPs, 1 mM primers, and 5 units of Taq polymerase (Perkin Elmer) in a total volume of 100 μl in an amplifying thermocycler (Perkin Elmer). The DNA sequence of all PCR products was confirmed by the dideoxy chain termination method of Sanger et al. (12) after cloning of the fragment into M13.

Cloning of N4SSB and Construction of Expression Plasmids—N4 DNA HpaD fragments were isolated by electrophoresis on a 1% vertical agarose gel (7), and the N4 HpaD fragment was isolated by electroelution and inserted into the Smal site of pSK232-8 (provided by J Urgen Brosius, Mount Sinai School of Medicine) (13). The ability of cloned DNA fragments to rescue phage carrying the N4SSBam7 mutation was determined as described previously (14).

The DNA fragment containing the N4SSBam7 open reading frame (ORF) and the distal 100 noncoding bases was amplified by PCR using N4am7 DNA as a template and the following oligonucleotides as primers.

| Primer       | Sequence                        |
|--------------|---------------------------------|
| N4SSB-N      | 5'-GGATTCTGCTGCTGCTGAGGAAATTCG-3' |
|              | EcoRI valine                    |
| N4SSB-C      | 5'-CAAGCTTCAGGCAGTTTCATTCGATTTTGGC-3' |
|              | HindIII                         |

N4SSB-N generates an additional valine residue as the second amino acid (underlined). After confirmation of the DNA sequence, the desired DNA fragment was inserted into pT5 (provided by Steve Eisenberg, Synergen) to yield pMC3, which carries the N4SSBam7 ORF under T7 RNA polymerase promoter control. Clones expressing the wild-type ORF were not recovered by this procedure (see “Results”).

To clone the wild-type N4SSB ORF, a DNA fragment carrying the ShinoDalgarno (SD) sequence present in pT5 and the amino- and terminal region of the N4SSB gene (120 amino acids) was generated by PCR, using N4 DNA and the following oligonucleotides as primers.

| Primer       | Sequence                        |
|--------------|---------------------------------|
| N4SSB-X1     | 5'-GTTCGGACGGAAAAAAATGCAATTATGCG-3' |
|              | XbaI                             |
| N4SSB-B1     | 5'-CAAGCTTCAGGCAGTTTCATTCGATTTTGGC-3' |
|              | HindIII                         |

The PCR fragment was restricted with XbaI and HindIII and inserted into the same sites of mp19 to verify the DNA sequence. The XbaI/BstBI fragment (N4SSBam7) was used to replace the corresponding fragment (carrying the am7 mutation) in pMC3, yielding pMC5. Finally, pMC5 was treated with BglII and XbaI to release the fragment containing the T7 RNA polymerase promoter. This fragment was replaced by a 66-base-long DNA fragment containing the T7 minimal promoter and lac operator, isolated from pET-11a (Novagen) by restriction with the same enzymes, to yield pMC6. N4SSB was purified as described (5) with some modifications. Deletion mutants were generated by oligonucleotide-directed, site-specific mutagenesis of pMC6.

Isolation and Analysis of Intracellular DNAs, Measurement of N4 DNA, and Late RNA Synthesis—E. coli W3350pcB (DE3)pLysE bearing the N4SSB expression vector pMC6 was grown at 37 °C to A600 = 0.4 in minimal salt medium (15) supplemented with 0.2% casamino acids (Difco) and 0.01 mg/ml thiamine. Cells were collected by centrifugation and resuspended in fresh medium. After a 15-min preincubation and a 30-min induction with 0.4 mM IPTG, cells were infected with N4am7 phage at a multiplicity of infection of 10. At different times after infection, 100 μl of cells were removed and processed as described (5).

To measure N4 DNA synthesis, the reaction was restricted with the indicated enzymes. After a 15-min preincubation and a 30-min induction with 0.4 mM IPTG, cells were infected with N4am7 phage at a multiplicity of infection of 10. At different times after infection, 50 μl of cells were removed and processed as described (5).

Labeling of Proteins after Induction—Cells were grown as described above. After induction with 2 mM IPTG for 30 min at 37 °C, cells (1 ml) were incubated for 90 min in the presence of 200 μM rifampicin at 37 °C, and proteins were labeled with 10 μCi of [35S]methionine (Amersham Corp.) or Tran35S-label (ICN) for 5 min at 37 °C. Cells were collected by centrifugation and processed as described previously (16).

N4SSB Activation of N4 DNA Recombination—E. coli W3350pcB (DE3)pLysE bearing wild-type or mutant N4SSB expres-

2 A. Miller, M. Choi, A. Glucksmann-Kuis, X. Dai, and L. B. Rothman-Denes, submitted for publication.

3 G. Lindberg, unpublished data.
sulted in polypeptides smaller than N4SSB due to premature termination of translation caused by mutations within the coding sequence for N4SSB. The largest cloned fragment coded for a 28-kDa polypeptide (pMC628), missing the carboxyl-terminal 35 amino acids due to the deletion of two adenines in a run of adenines at positions 688–691 (Fig. 1). These mutations occurred even in the absence of active T7 RNA polymerase. In this case, expression of N4SSB originated from cryptic E. coli RNA polymerase promoters present between the T7 promoter and Shine-Dalgarno sequence of N4SSB (data not shown). These results indicate that expression of N4SSB is lethal to E. coli. The N4 am7 ORF was cloned (pMC3) to overcome this problem (see "Materials and Methods"). The successful isolation of pMC3 indicates that low levels of N4SSB protein are lethal to the cell.

Cloning of the wild-type N4SSB ORF (pMC6) was successfully achieved using a tightly regulated system in which the N4SSB gene is under the control of a T7 RNA polymerase promoter and the lac operator. In addition, the recombinant plasmid was introduced into a host strain carrying the pcnB mutation, which reduces the copy number of pBR322 derivatives (8), and pLysE, which synthesizes the T7 lysozyme and is an inhibitor of T7 RNA polymerase. Fig. 2 shows the expression of N4SSB in E. coli W3350pcnB(DE3)/pLysE, which was used as a host for N4 phage infection to test the effect of cloned N4SSB on N4 DNA recombination, N4 DNA replication, and N4 late transcription in vivo, and in BL21(DE3)/pLysE, which was used for the overexpression and purification of cloned N4SSB. The size of the N4SSB protein produced by the T7 RNA polymerase-directed, expressing clone was the same as that from N4-infected cells. The amount of N4SSB is higher in E. coli BL21(DE3)/pLysE than in E. coli W3350pcnB(DE3)/pLysE due to a higher copy number of pMC6 in the former strain. The ability of the cloned and expressed N4SSB protein to complement N4 am7 for N4 DNA replication was examined. Fig. 3 (left panel) shows the rate of [3H]thymidine incorporation into DNA after N4 am7 infection of the following cells: suppressor (W3350 supF), non-suppressor (W3350), W3350pcnB(DE3)/pLysE carrying pMC6, or pMC628 without or with preincubation with 0.4 mM IPTG for 30 min. Even though the rate of [3H]thymidine incorporation in cells expressing cloned N4SSB was lower than in suppressor-containing cells, it was 4-fold higher than in noninduced or N4SSB28-expressing cells. The increased rate of thymidine incorporation occurred in N4SSB-expressing cells when cells had been preincubated with 0.2–0.5 mM IPTG for 20–45 min. The amount of N4SSB, synthesized from the T7-directed clone induced with 0.1–1 mM IPTG for 15–90 min, was found to be comparable to that produced in N4am7-infected suppressor-containing cells (data not shown).

Southern blot analysis was performed to test whether the increased rate of thymidine incorporation in N4SSB-expressing cells was due to active N4 DNA replication, recombination, or repair. Total intracellular DNAs (host chromosomal, N4 genomic, and plasmid DNAs) were prepared from wild-type
N4SSB-expressing cells that had been preincubated in the absence or presence of 0.4 mM IPTG for 30 min at three different times (8, 18, and 35 min) after N4am7 infection. DNAs were restricted with XbaI and SalI, and fragments were separated on an agarose gel, blotted, and hybridized to an excess amount of 32P-labeled genomic DNA (see "Materials and Methods").

The defect in all mutants discussed above can be explained by impaired single-stranded DNA binding activity and/or a differential involvement of the single-stranded DNA binding activity of the protein in recombination, replication, and activation of late transcription. To rule out this possibility, the N4SSB mutants were subjected to a gel shift assay to determine their ability to bind to single-stranded DNA. A single-stranded DNA oligomer containing one binding site (12-mer DNA) was used as a template in gel shift experiments. N4SSB mutants were expressed in BL21(DE3)λ lysE carrying pMC6 and in E. coli W3350 pcnB (DE3)λ lysE carrying pMC6 in the absence or presence of 1 mM IPTG. For experimental details, see "Materials and Methods." The arrowhead indicates the N4SSB polypeptide.

The results show that the N4SSB mutants were expressed to the same degree in the absence and presence of IPTG. The ability of N4SSB mutants to complement N4 DNA replication was measured. The results of these experiments are presented in Table II.

Deletion of the three carboxyl-terminal amino acids generated a protein (N4SSB263–265) active in supporting replication, but inactive in recombination or late transcription. Deletion of an additional residue (N4SSB262–265) abolished all three activities. These results suggest that the carboxyl-terminal region of N4SSB is required for interactions with the polymerase, recombination, and transcriptional machineries.

To determine the role of the carboxyl terminus, we generated two types of deletions by site-specific mutagenesis: carboxyl-terminal truncations and internal, in-frame deletions. The sequence of the mutant N4SSB ORFs in each plasmid was confirmed by double-stranded DNA sequencing. Mutant proteins were cloned for overexpression in W3350 pcnB (DE3)λ lysE. The size and amount of expressed mutant protein were determined, following IPTG induction and [35S]methionine labeling, by SDS-polyacrylamide gel electrophoresis and autoradiography. All mutants were of the expected size, were expressed to the same degree, and were as stable as the wild-type protein (Fig. 5).

The ability of N4SSB mutants to complement N4am7 for N4 late transcription, to support N4 DNA recombination, and to activate N4am7 DNA replication was measured. The results of these experiments are presented in Table II.
irradiation at 300 ergs/mm². Single-stranded DNA-bound N4SSB complexes were analyzed on a native polyacrylamide gel (Fig. 6). N4SSB binding to a 12-mer produces a major retarded species and a minor species due to protein-protein interactions. N4SSB binds more efficiently than the wild type to the single-stranded 12-mer, but fails to form the second complex. These and other results (data not shown) indicate that N4SSB is deficient in N4SSB-N4SSB interactions.

**DISCUSSION**

We have succeeded in sequencing and cloning the gene for N4SSB, a protein required for viral DNA replication, activation of late transcription, and, as we demonstrate in this paper, phage recombination. N4SSB is 265 amino acids in length, and no sequence similarity to other single-stranded DNA-binding proteins is evident. Specifically, the acidic carboxyl-terminal region present in several single-stranded DNA-binding proteins is absent in the predicted N4SSB sequence (19). However, both N4SSB and T4 gp32 contain a series of similarly spaced aromatic and charged residues in the first 130 amino acids (20).

Our inability to clone the N4SSB gene suggests that it is highly toxic to *E. coli*. While it is not yet clear what the determinants of lethality are, two SSB functions might be involved: its single-stranded DNA binding activity and its ability to activate RNA polymerase at the N4 late promoters. Successful cloning of N4SSB required a tightly regulated expression system encompassing (a) deletion of weak *E. coli* promoters present upstream of the N4SSB translational start site, (b) use of the chromosomal *pcnB* mutation to reduce the plasmid copy number, (c) introduction of the T7 lysozyme carried on plasmid pLysE to inhibit T7 RNA polymerase, and (d) introduction of the lac operator sequence immediately downstream of the T7 promoter to prevent T7 RNA polymerase from binding to its promoter until IPTG is added.

The cloned and expressed protein was able to complement N4am7 in vivo for N4 DNA replication and N4 late transcription. In addition, expression of wild-type N4SSB from T7-directed expressing clones increased N4 DNA recombination 10⁴–10⁵-fold, indicating that N4SSB is required for N4 DNA recombination.

Even though cloned N4SSB was able to complement N4am7 for N4 DNA replication and N4 late transcription, the level of activation did not reach those observed after N4am7 infection of suppressor-carrying cells. We have considered several alternative explanations that can account for the lower level (20%) of replication and late transcription activation in cloned N4SSB-expressing cells. N4am7 is not a dominant negative...
constructed. The N4SSB protein and the expected products

from pMC8am7 in E. coli W3350 pcnB(DE3)/pLysE.4 These results suggest that the am7 mutation does not affect the expression of the two downstream genes. The ability of cloned N4SSB and the two downstream gene products to complement N4am7 for N4 DNA replication and N4 late transcription was examined (data not shown). The levels of both activities were similar to those observed when only N4SSB was expressed. We suspect that the inability of cloned N4SSB to fully activate DNA replication and late transcription is due to the timing of N4SSB expression during N4 development under these conditions. The lower level of late transcription might also be the result of lower levels of template.

The N4SSB expression vector pMC6 was able to rescue N4am7, while pMC6am was not. Increased expression of wild-type N4SSB from expressing clones increased N4 DNA recombination 104-105-fold. This observation indicates that N4SSB from expressing clones increased N4 DNA recombination 104-105-fold. This observation indicates that N4SSB plays an essential role in N4 DNA recombination. Two other lines of evidence indicate that N4SSB is essential for N4 recombination independently of its requirement for N4 DNA replication. First, N4SSB(Δ263-265), supports DNA replication, while it is defective in activating N4 recombination (Table II). Second, two additional N4SSB mutants (N4SSB(Δ264-265,K259R) and N4SSB(Δ245-259), although deficient in supporting replication, can activate recombination to wild-type or nearly wild-type levels. N4 DNA recombination is independent of host recombination genes, suggesting that N4 encodes its own recombination functions.5 T4 gp32 is also required for T4 DNA recombination.6 In contrast, E. coli SSB activates host DNA recombination; its absence in E. coli ssb mutant strains reduces recombination only 7-fold (21).

N4SSB and E. coli SSB differ from other well characterized single-stranded DNA-binding proteins in that, in addition to

| Host            | N4 phage infection | IPTG |
|-----------------|--------------------|------|
|                 | 0 μM               | 0.02 μM | 0.2 μM | 1 μM |
| W3350           | N4am7              | 4.0 x 10^6 | ND* | 6.0 x 10^6 | 2.0 x 10^7 |
| W3350supF       | N4am7              | 4.0 x 10^12 | ND | 2.0 x 10^12 | 4.0 x 10^7 |
| W3350pcnB(DE3)/pLysE + pMC6 | N4am7 | 4.0 x 10^7 | 1.0 x 10^8 | 1.0 x 10^9 | 8.0 x 10^13 |
| N4              | 1.2 x 10^13 | 1.2 x 10^1 | 1.3 x 10^13 | 1.7 x 10^13 |
| W3350pcnB(DE3)/pLysE + pMC6am | N4am7 | 1.0 x 10^7 | 1.0 x 10^7 | 4.0 x 10^7 | 2.0 x 10^7 |
| N4              | 2.0 x 10^13 | 2.0 x 10^13 | 2.0 x 10^13 | 1.5 x 10^13 |
| W3350pcnB(DE3)/pLysE + pMC3  | N4am7 | 1.0 x 10^7 | 3.0 x 10^7 | 2.0 x 10^7 | 2.0 x 10^7 |
| N4              | 1.8 x 10^13 | 2.0 x 10^13 | 2.0 x 10^13 | 2.5 x 10^13 |

* ND, not determined.

For experimental conditions, see “Materials and Methods.”

For experimental details, see “Materials and Methods.”

FIG. 5. Expression of cloned mutant N4SSB proteins in E. coli W3350pcnB(DE3)/pLysE carrying wild-type N4SSB (wt) or the indicated N4SSB mutants. Expression plasmids were incubated in the absence or presence of 1 μM IPTG, and the expressed proteins were labeled and analyzed as described under “Materials and Methods.”

FIG. 6. Binding of N4SSB and N4SSB(Δ262-265) to single-stranded DNA. Reaction mixtures containing an excess of labeled 32-mer oligonucleotide and increasing concentrations of wild-type (wt) N4SSB or N4SSB(Δ262-265) were treated and applied to an 8% native polyacrylamide gel as described under “Materials and Methods.” Labeled free probe is not shown.

mutation. The N4am7 mutation could exert polarity, affecting the expression of downstream gene products that might be required for N4 DNA replication and late transcription. The downstream region (2 kb) of the N4SSB-coding region was sequenced. Two ORFs (185 and 147 amino acids in length) are present. An expression plasmid (pMC8) carrying the wild-type N4SSB or am7 allele and the two downstream ORFs was constructed. The N4SSB protein and the expected products from the two downstream ORFs were expressed in E. coli W3350pcnB(DE3)/pLysE. These proteins were also expressed

4 M. Choi, unpublished data.
5 S. Spellman and L. B. Rothman-Denes, unpublished data.
6 G. Mosig, personal communication.
their involvement in replication and recombination, they are transcriptional activators (6, 22). N4SSB and E. coli SSB are unique among transcriptional activators in that they do not bind to a specific double-stranded DNA site for activation to occur. How do they accomplish their transcriptional activation tasks in the absence of a cognate DNA-binding site? We have shown that E. coli SSB is an activator of the N4 virion-encapsulated DNA-dependent RNA polymerase by providing the correct DNA structure, a DNA hairpin on the template strand, for N4 virion RNA polymerase recognition (22, 23). DNA-binding sites for transcriptional activators serve at least two functions: 1) to increase the local concentration of the activator at its target and/or 2) to position the activator so as to make proper contacts with the transcriptional machinery. A DNA-binding site for N4SSB as a transcriptional activator might not be required since it is expressed at high levels during infection: ~11,000 molecules of N4SSB/cell (9–18 μM) (5). Indeed, we have recently proposed that an N4SSB-E. coli RNA polymerase complex forms, which then binds to N4 late promoters (24). Preliminary results indicate that the single-stranded DNA binding activity of N4SSB is not required for transcriptional activation.8

The isolation of N4SSB mutants differentially affected in DNA replication, recombination, and activation of late transcription (Table II) suggests that different determinants of N4SSB are important for these different activities. The isolation and characterization of additional N4SSB mutants specifically affected in DNA replication, recombination, activation of late transcription, single-stranded DNA binding, and cooperativity are required to understand the role of N4SSB in these processes.

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