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GLUTATHIONE S-TRANSFERASE-SSPA FUSION BINDS TO *E. coli* RNA POLYMERASE AND COMPLEMENTS Δ*sspA* MUTATION ALLOWING PHAGE P1 REPLICATION

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Bacteriophage P1 is unable to form plaques on *E. coli* hosts lacking a functional *sspA* gene. However, *sspA* mutants can be infected by P1, resulting in the synthesis of P1 early gene products and accumulation of P1 DNA, but without P1 late gene product formation or host lysis. Overexpression of the stringent starvation protein (SspA) as a glutathione-S-transferase fusion results in complementation of the *sspA* mutation and production of viable viral particles as in *sspA*$^+$ strains. This suggests that the GST-SspA protein functions *in vivo* in a similar manner as native SspA with respect to P1 replication. Here, evidence is presented that shows that SspA binds to RNA-polymerase. This supports the notion that SspA is involved in P1 replication since it is known that P1 requires host RNA-polymerase activity to replicate and this suggests a mechanism by which P1 redirects *E. coli* RNA-polymerase specificity from P1 early to P1 late genes.

The lysogenic conditions of the *E.coli* temperate bacteriophage P1 have been extensively studied (1,2) and the regulation of the lytic growth cycle is beginning to be defined. P1 replication depends on a rifampicin-sensitive host RNA-polymerase (RNAP) and a functional phage-encoded gp10 gene product for activation of the late P1 promoter, P_S (3). P1 gp10 may function to modify the specificity of host RNAP, (E) to recognize phage promoter sequences which differ from host promoter sequences (3,4). This redirecting of RNAP is a requirement for bacteriophage, such as P1, which do not encode their own RNAPs. Evidence presented here and elsewhere (6,7) suggest that the SspA protein of *E. coli* is also involved in P1 replication.

The *E.coli* stringent starvation protein (SspA) was originally isolated with RNAP (5) and is the first gene product in the starvation-inducible *sspA/sspB* operon (6,7). Although the *sspA* promoter sequence, P_{ssp}, has significant sequence homology with Tanaka *et al.* type III promoters (e.g. P_{fic}, P_{katE}, P_{mcb}, P_{bolA}) (8) recognized by a stationary phase form of RNAP bound to the *katF/rpoS* gene product σ^S ($E\sigma^{38}$), expression from P_{ssp} is neither *rpoS* nor *rpoH* dependent (7). Expression of the *sspA* gene has been shown to be *relA* dependent and required for the expression of a number of P1 lytic genes (6). The function of *sspB* is unknown.

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Inhibition of P1 replication in *sspA* mutants results in the absence of some (and perhaps all) P1 late gene products. In *sspA* mutants, P1 early gene products are synthesized throughout the infection cycle extending to late time points during which wild-type strains are synthesizing late gene products (6). P1 DNA accumulation is known to occur in *sspA* mutants but apparently is not packaged due to the absence of P1 late gene products (such as phage heads and tails) (6).

EXPERIMENTAL METHODS

E.coli strains DH5αF', EAB17 and MW14333 and construction of plasmids pMDW77, pGEX-3X-SspA has been reported previously (6, 7). The *sspA* gene was isolated on a 1349 bp *Hin*cII fragment from pMDW77, and inserted into pSELECT-1 (9), cleaved by *Sma*I digestion and treated with calf intestinal phosphatase (CIP). The ligation mixture was used to transform DH5αF' to ampicillin (amp) resistance; transformants were selected on SOC-amp plates and the orientation of the *sspA* insertion was determined by *Afl*III digestion. Site-directed mutagenesis was done to eliminate the *Nla*III site at base pair 935 with the *sspA* gene using a mutagenic 20 mer oligonucleotide in which TAC (935) was changed to TAT without changing the codon for tyrosine. The annealing reaction was performed with single-stranded pSELECT-*sspA* template DNA, amp repair oligonucleotide, phosphorylated mutagenic oligonucleotide primer and annealing buffers. The reaction mixture was used to transform BMH-71-18MUT5 (9). Mini-prep DNA from BMH-71-18MUT5 was transformed into DH5αF' and clones selected on SOC-Amp-Tet plates. The transformants were analyzed by DNA sequencing. The mutated *sspA* gene was isolated from pSELECT-*sspA* by *Eco*RI, *Bam*HI double digestion followed by *Nla*III digestion. The 992 bp *Nla*III-*Nla*III fragment containing the*sspA* was isolated and treated with T4 DNA polymerase to produce a blunt-ended fragment. This fragment was ligated with *Sma*I-digested and CIP-treated pGEX-3X (9). The ligation mixture was used to transform competent DH5αF' cells followed by selection on SOC-Amp plates. The orientation of *sspA* was determined by digestion of pGEX-*sspA* by *Eco*RI and *Sma*I. Expresssion of GST-SspA fusion protein was under the control of the IPTG-inducible *tac* promoter.

GST-SspA was purified from cell lysates by glutathione Sepharose-4B affinity chromatography by elution with 15 mM glutathione in 50 mM Tris-HCl, pH 7.5. Fractions containing GST-SSP were combined, dialyzed against PBS to remove glutathione and re-purified on the glutathione affinity column to a final purity of approximately 90%. The yield of fusion protein was 4 mg/l from a culture of 10 g/l *E. coli* DH5αF'cell dry weight.

RNA polymerase binding assays were carried out following the method of Lesley and Burgess (10). 15 μg of GST-SspA fusion protein was allowed to react with core-RNAP (9.6 μg) at 30°C for 30 min in 20 mM Tris-HCl, pH 7.9, 0.2 M NaCl, 1 mM EDTA and 0.1 mM DTT (10) in a total volume of 50 μl. Following incubation, the mixture was loaded onto a Sephadex G-75 column (Biorad) equilibrated with the above buffer at 4°C, and eluted in 0.5 ml fractions. Fraction volume was reduced to 0.2 ml by speed vacuum and trichloroacetic acid precipitation. Proteins were resuspended in SDS-PAGE sample buffer and analyzed by 12% PAGE.

RESULTS

During attempts to over-express SspA in the form of a glutathione-S-transferase fusion (GST-SSP) (7), it was observed that the expression of GST-SspA from pGEX-3X in an *sspA* mutant, EAB17, which was unable to produce any plaques in a P1 lysate assay (11) complemented the Δ*sspA* mutation when cloned in the correct orientation. Complementation of Δ*sspA* produced a comparable number of plaques as the wild type strain BW14333 (ssp^+) (Table 1). This suggests that the GST-SspA fusion protein has the same activity *in vivo* as native SspA in the P1 lysis assay (6).

TABLE 1

Plaque Formation by *ssp* Mutants in the P1 Lysate Assay

Strain	Plasmid	P1 Plaques Formed (10^{-4})
BW14333 (*sspA*$^+$)	none	211
EAB17 (*sspA*)	none	none
EAB17 (*sspA*)	pMDW77	297
EAB17 (*sspA*)	pGEX-3X	none
EAB17 (*sspA*)	pGEX-3X-*sspA*	200

Direct biochemical evidence that *sspA* encodes an RNAP binding factor used by P1 to alter the recognition of *E.coli* RNAP was obtained by purification of GST-SspA by glutathione Sepharose affinity chromatography. Chicken polyclonal antibodies (12) to GST-SspA were used to follow fusion protein purification using western blotting along with a quantitative ELISA using goat-anti-chicken IgG (H + L) conjugated with horseradish peroxidase. GST-SspA was found to bind to either core- or holo-RNAP *in vitro* (Figure 1). Although the binding of GST-SspA to holopolymerase (Figure 1B.) differs from earlier reports (5), this cannot be stated with certainty since preparations of holo polymerase may be contaminated with core polymerase. GST alone appears to be eluting with RNAP, however its presence does not correlate with peak fractions of RNAP and the association observed probably results from overloading of GST or an elution rate that is incompatable with the column. The peak fractions of GST-SspA correlate with the peak fractions of RNAP confirming that SspA is an RNAP binding protein (5).

CONCLUSIONS

This direct biochemical evidence suggests that SspA may be a sigma factor-like component of a stationary phase form of *E.coli* RNAP such as the *katF/rpoS* gene product, the synthesis of which is increased prior to the onset of nutrient starvation during the stationary phase of *E.coli* growth (8, 13 - 17). It also raises the possiblility that SspA expression is positively regulated by ppGpp as has been recently shown for the *katF/rpoS* gene product (18). The RNAP-GST-SspA complex may function to alter polymerase recognition for binding to some P1-encoded promoters resulting in plaque formation in Δ*sspA* hosts. Involvement in P1 replication and RNAP binding are the only known functions of SspA other than evidence that *sspA* mutants have significantly altered global regulation of protein synthesis in response to amino acid, carbon, nitrogen or phosphate starvation (7).

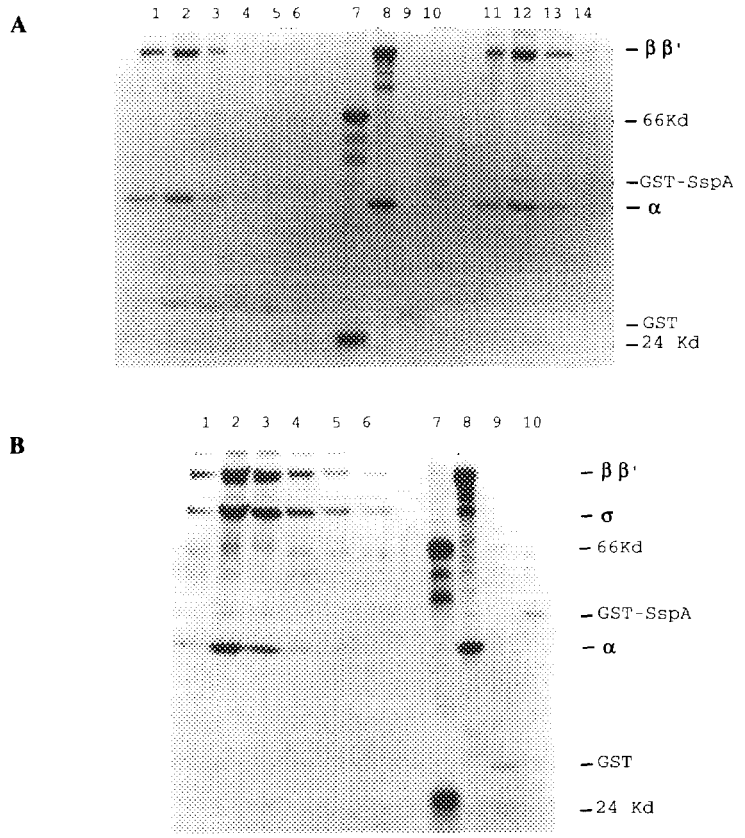

Fig. 1. Analysis of *in vitro* binding of GST-SspA to *E.coli* RNA Polymerase. **A**. Binding of GST-SspA to Core-RNAP. Lanes 1-6, successive fractions containing RNAP following incubation with GST; lane 7, molecular weight markers; lane 8, holo-RNAP; lane 9, GST; lane 10, GST-SspA; lanes 11-14, successive fractions containing RNAP following incubation with GST-SspA. **B**. Binding of GST-SspA to Holo-RNAP. Lanes 1-6, successive fractions containing Holo-RNAP following incubation with GST-SspA.; lane 7, molecular weight standards; lane 8, holo-RNAP; lane 9, GST; lane 10, GST-SspA.

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