Purification and Some Properties of a Factor (S) Blocking the Synthesis of Covalently Linked Complementary Sequences by Deoxyribonucleic Acid Polymerase I†

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ABSTRACT: A protein factor, S, has been purified to greater than 90% homogeneity from *Escherichia coli*. S factor blocks the accumulation of DNA with covalently linked, complementary (clc) sequences during the DNA polymerase I mediated copying of some defined DNAs. It is also partially effective when *E. coli* DNA is the template. The factor must be present during the course of synthesis to be effective; it does not remove clc linkers once these are formed. It is free of demonstrable nucleolytic activity. The factor activity behaves as a globular protein of molecular weight 26,000 during Sephadex chromatography, and the protein has an apparent molecular weight of 11,500 in a denaturing solvent. S factor is distinguishable from known single-stranded DNA binding proteins, and proteins which stimulate RNA polymerase. The model proposed is that S factor blocks the strand switching or reversing by DNA polymerase.

The enzyme DNA polymerase I of *Escherichia coli* will copy various DNAs extensively *in vitro*. Although natural DNAs can be copied with great fidelity (Goulian and Kornberg, 1968; Goulian *et al.*, 1968) the product of extensive, net-fold synthesis is not biologically active, and consists of branched structures. These structures rapidly reanneal after denaturation (Schildkraut *et al.*, 1964). We have termed these structures covalently linked, complementary (clc[1]) sequences (Morgan and Paetkau, 1972) as being a more descriptive term than "snap-back" or "renaturing." Defined DNAs also give rise to clc structures when copied extensively *in vitro* with DNA polymerases (Paetkau, 1969; Harwood and Wells, 1970).

Polymerase I appears to be similar in its synthetic mode to other known DNA polymerases (Gefter, 1972). One should expect that in a properly reconstituted system, extensive copying would not result in clc sequences. An earlier report described a heat-labile activity which would block the accumulation of clc sequences during the copying of the defined DNA, $d(T\text{-}G)_n d(C\text{-}A)_n$ (Paetkau, 1969). More recently, we have outlined various assays for clc structures (Morgan and Paetkau, 1972; Coulter *et al.*, 1974) and described the synthesis of some defined DNAs free of clc sequences. In the latter reference, a crude preparation of DNA polymerase was shown to contain an activity, termed S factor (for separability of strands), which was apparently not a common endonuclease. S factor blocked the synthesis of clc structures during the polymerase-mediated copying of both $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$. This paper describes the purification of S factor and some physical and enzymatic properties of this protein.

Experimental Section

Materials. Gel electrophoresis materials were obtained from Eastman Kodak Co., DEAE-cellulose (DE-23) was obtained from Whatman Industries, Sephadex G-25 (20–80 μ), G-50 (20–80 μ), G-75 (40–120 μ), Blue Dextran 2000, ovalbumin, and chymotrypsinogen A from Pharmacia Fine Chemicals, sperm-whale myoglobin and cytochrome *c* were gifts from Dr. J. Seehafer, R17 phage was a gift from Mrs. L. Frost, PM2 DNA was a gift from Dr. A. R. Morgan, and unfractionated yeast tRNA was a gift from Dr. C. J. Smith.

E. coli DNA was isolated by the method of Marmur (1963) and furthr purified by heating to 47° for 15 min in 0.1% sodium dodecyl sulfate followed by gel exclusion chromatography on Agarose 5m with 10 mM Tris-Cl (pH 8.0)–0.1 mM EDTA. T4 DNA was purified by phenol extraction and dialysis.

Other materials were as already described (Morgan *et al.*, 1974; Coulter *et al.*, 1974).

Methods. Nuclease assays were based either on conversion of radioactively labeled DNAs to Cl_3CCOOH soluble radioactive material, or by conversion of covalently closed circular PM2 DNA to the nicked form. The two strands of the nicked, but not the closed, form of this DNA will separate upon heat denaturation. After cooling, the fluorescence assay described earlier (Coulter *et al.*, 1974) was used to determine the amount of nicked PM2 DNA and therefore the double-strand specific endonuclease activity (A. R. Morgan, unpublished).

Detection of clc-DNA was by the methods described in Coulter *et al.* (1974), using 2 mM Tris-Cl–0.2 mM EDTA buffer for defined DNAs and 20 mM K_3PO_4–0.2 mM EDTA buffer for natural DNAs. S factor activity was routinely determined by measuring the clc-DNA content of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ synthesized as described in Coulter *et al.* (1974). The reaction mixture contained 30 mM potassium phosphate (pH 7.4), 12 mM $MgCl_2$, 2 mM dithiothreitol, 1.3 mM each of dATP, dCTP, dGTP, and TTP, 0.2 OD_{260} of $d(T\text{-}G)_n d(C\text{-}A)_n$ template, 0.5 OD_{260} tRNA to inhibit endonuclease I in crude S factor preparation (Lehman *et al.*, 1962), pancreatic DNase I to stimulate synthesis, as indicated, and S factor as indicated. Incubation was at 37°, for the times indicated. A unit of S factor activity was taken to be the reduction of clc-DNA content by 50%.

Apparent molecular weights of S factor were determined by chromatography on calibrated Sephadex G-75 columns

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[1] Abbreviations used are: clc-DNA, DNA with covalently linked, complementary sequences; S factor, separability of strands factor.

(Andrews, 1970) and by sodium dodecyl sulfate containing acrylamide gel electrophoresis (Shapiro *et al.*, 1967).

Fraction 4 DNA polymerase was obtained from *E. coli* B essentially as described by Richardson *et al.* (1964). The ammonium sulfate precipitate was dissolved to yield a protein concentration of 40 mg/ml. This was the starting material for the purification of both DNA polymerase (Jovin *et al.*, 1969) and S factor.

RNA polymerase assays were performed at 37° in a mixture containing 0.04 M Tris-Cl (pH 8.0), 4 mM $MgCl_2$, 0.8 mM $MnCl_2$, 10 mM β-mercaptoethanol, 40 mM KCl, 0.4 mM [^{3}H]CTP (100 cpm/pmol), 0.4 mM each of ATP, GTP, and UTP, 0.016 or 0.04 OD_{260} T4 DNA, and 2 μg/ml of fraction VI RNA polymerase (containing σ subunit) (Paetkau and Coy, 1972). Portions were removed at various times and RNA synthesis was determined by incorporation of label into acid-insoluble material.

Other methods were as described earlier (Morgan *et al.*, 1974; Coulter *et al.*, 1974).

Results

1. *Purification of S factor.* All operations were at 0–4° unless otherwise indicated. The starting material was fraction 4 DNA polymerase (see Methods), protein concentration 40 mg/ml.

A. *DEAE-Cellulose chromatography* (*1*) removed some nucleotidic material; 1 ml of 1 M potassium phosphate (pH 6.5) was added to 20 ml of fraction 4 (obtained from 1 lb of frozen cells) to lower the pH and to increase the phosphate concentration to 0.07 M. The sample was clarified by centrifugation and applied under moderate pressure to a DEAE-cellulose column (0.63 cm^2 × 7 cm) equilibrated with buffer A (0.2 M potassium phosphate (pH 6.5)–0.01 M β-mercaptoethanol–0.1 mM EDTA). The flow rate was 0.3–0.4 ml/min. The protein flow-through was pooled as fraction 5.

At this stage of purity, most of the S factor activity was completely excluded from Sephadex G-75. Autolysis, as described below, quantitatively converted it to a partially included form. The autolysis was successful only after low molecular weight inhibitory components had been removed, and free Mg^{2+} added, as follows. (i) Fraction 5 (26 ml) was chromatographed on Sephadex G-25 (4.9 cm^2 × 38 cm) equilibrated with buffer A. The flow rate was 0.5 ml/min and 12.6-ml fractions were collected. The excluded protein peak had an OD_{280} to OD_{260} ratio of 1.49, compared to 0.68 for fraction 5. (ii) Concentration of the excluded material from Sephadex G-25 (38 ml) was performed in the Model 52 Amicon ultrafiltration cell using a PM 10 membrane at 45 psi nitrogen. The final volume was 6 ml. (iii) Autolysis of the concentrated material at room temperature followed clarification by centrifugation and addition of $MgCl_2$ to 4 mM. The extent of autolysis was monitored by measuring the rate of acid solubilization of 0.52 OD_{260} [[^{3}H]T,[^{14}C]dC]-d(T-G)$_n$·d(C-A)$_n$ added to a portion of the sample. Autolysis was stopped at the time of total acid solubilization, which required from 60 to 120 min, depending on the sample.

B. *Sephadex G-75 chromatography* of the autolyzed sample followed directly. The sample (6 ml) was centrifuged and then applied under gravity to a column of Sephadex G-75 equilibrated with buffer A (Figure 1). The S factor activity was removed from the bulk of the protein, eluting at a position corresponding to a globular protein of molecular weight 26,000. The fractions containing S factor activity were pooled (25 ml) and passed over a Sephadex G-25 column (4.9 cm^2 × 38 cm) equilibrated with buffer B (10 mM Tris-Cl (pH 8.0)–

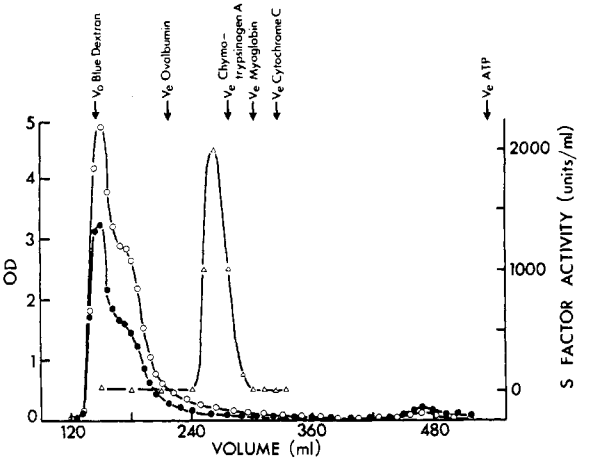

FIGURE 1: Sephadex G-75 chromatography of fraction 5 S factor; 6 ml of autolyzed fraction 5 was applied to a Sephadex G-75 column (4.91 cm^2 × 95 cm) equilibrated with 0.2 M potassium phosphate (pH 6.5)–10 mM β-mercaptoethanol–1 mM EDTA. The flow rate was 15 ml/hr and 5.2-ml fractions were collected. S factor activity was measured with the standard d(T-G)$_n$·d(C-A)$_n$ assay (see Methods). The elution positions of Blue Dextran 2000, ovalbumin, chymotrypsinogen A, sperm-whale myoglobin, cytochrome *c*, and ATP were determined in a separate run: (○) OD_{280}; (●) OD_{260}; (△) S factor activity.

0.1 mM EDTA). The excluded protein was concentrated by lyophilization and dissolved in 3 ml of water as fraction 6.

Fraction 6 contained both tRNA-sensitive DNase, assumed to be endonuclease I (Lehman *et al.*, 1962), and tRNA-insensitive DNase. At the dilution of fraction 6 which reduced the clc content by 50% in the assay (1 unit/ml), the tRNA-insensitive nuclease would have completely degraded the input template in 25–50 hr.

The S factor also contained nucleotidic material at this stage. The nucleotidic material, which was not removed by chromatography on either Sephadex or DEAE-cellulose, was apparently oligo[d(A-T)], based on the following evidence. Fraction 6 served as a template for DNA polymerase, incorporating [^{3}H]TTP but not [^{14}C]dCTP into acid-insoluble material. The incorporation of TTP occurred only with dATP present. The polymerase product was 100% clc, a property of d(A-T)$_n$ (Morgan and Paetkau, 1972). Incubation with $MgCl_2$, or exonuclease III, failed to destroy this template activity. The elution profile of the template activity was coincident with that of S factor activity when fraction 6 was chromatographed on DEAE-cellulose. The way in which the apparent oligo[d(A-T)] could be separated from S factor (below) indicates that it was of low molecular weight.

Unlike fraction 5, fraction 6 S factor led to a decrease, followed by an increase in clc content of product as the level of the factor was increased (Figure 2). This was accompanied by a decrease in the ratio of [^{14}C]dCTP to [^{3}H]TTP incorporated. These results are consistent with progressive increases in d(A-T)$_n$ synthesis as the fraction 6 factor was increased. This was not seen with fraction 5 factor, probably because the oligo[d(A-T)] template was degraded by nucleases during the early part of the assay.

C. *Urea–lithium chloride treatment* of fraction 6 was designed to dissociate S factor activity from the putative oligo[d(A-T)]. The solvent was based on the work of Traub and Nomura (1968); 1 ml of 8 M urea–8 M lithium chloride–12 mM β-mercaptoethanol was added to 1 ml of fraction 6, and the mixture incubated in an ice–water slurry for 36 hr. The solution was centrifuged and applied under gravity to a Sephadex

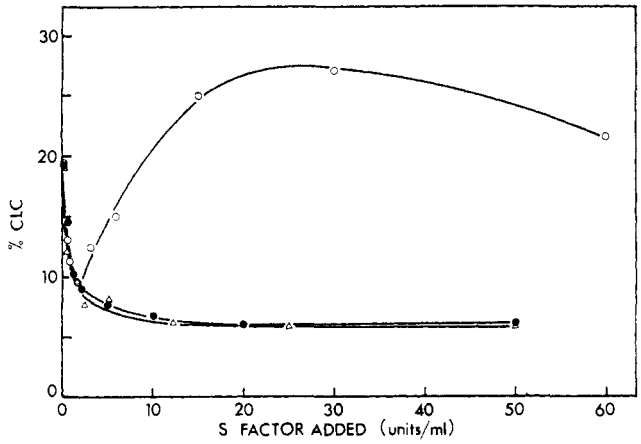

FIGURE 2: Titration curves of S factor fraction 6, 7, and 8. S factor activity was determined in the $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ assay system. Time of DNA synthesis was 5 hr, and 25 ng/ml of pancreatic DNase I was present. Fractions 6 and 7 assays contained 0.5 OD_{260} yeast tRNA: (○) fraction 6; (●) fraction 7; (△) fraction 8.

G-50 column 0.635 $cm^2 \times 55$ cm) equilibrated with buffer C (4 M urea–4 M LiCl–10 mM Tris-Cl (pH 7.5)–6 mM β-mercaptoethanol). After the sample was on the column, the flow rate was maintained at 2.3 ml/hr and 0.9-ml fractions were collected. The excluded peak, 5.4 ml, was dialyzed for 15 hr against 500 ml of buffer D (6 M urea–2 M LiCl–10 mM Tris-Cl (pH 7.5)–6 mM β-mercaptoethanol). This buffer was then diluted by pumping buffer E (0.5 M KCl–5 mM sodium phosphate (pH 7.2)) into the dialysis chamber at 40 ml/hr for 48 hr. The protein was passed over a Sephadex G-25 column (1.33 $cm^2 \times 45$ cm) equilibrated with buffer B, and the excluded peak was concentrated by lyophilization and dissolved in 1.5 ml of water as fraction 7. At this stage, no oligo [d(A-T)] was demonstrable by the $d(A\text{-}T)_n$ synthesis assay.

D. *DEAE-cellulose chromatography II* removed residual nuclease activity from S factor (Figure 3). The applied protein was eluted with a NaCl gradient. Fractions containing S factor activity were pooled (6 ml) and passed over a Sephadex G-25 column (1.33 $cm^2 \times 45$ cm) equilibrated with buffer B, and the excluded peak was concentrated by lyophilization and dissolved in 1 ml of water as fraction 8.

The purification procedure is summarized in Table I.

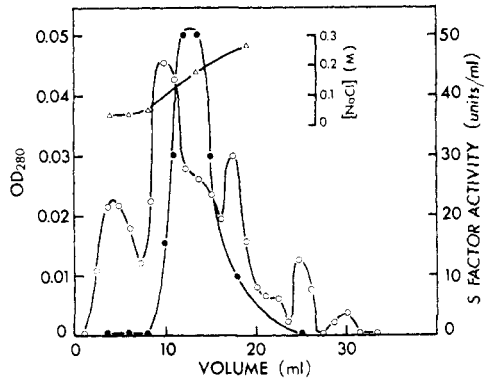

FIGURE 3: DEAE-cellulose chromatography II; 1 ml of fraction 7 was diluted with 4 ml of 50 mM Tris-Cl (pH 8.0) and applied to a DEAE-cellulose column (0.18 $cm^2 \times 7$ cm) equilibrated with buffer F (50 mM Tris-Cl (pH 8.0)–10% glycerol). The column was washed with 2 ml of buffer F, and the protein was eluted with a linear gradient of 15 ml of buffer F plus 0.03 M NaCl and 15 ml of buffer F plus 0.45 M NaCl. The flow rate was 7.8 ml/hr, and 1.3-ml fractions were collected: (○) OD_{280}; (●) S factor activity, determined by the fluorescence assay on $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$; (△) NaCl molarity, determined refractometrically.

TABLE I: Purification of S Factor.[a]

	Total Protein (mg)	Total Units	Specific Activity (units/mg)	Yield[d] (%)
4. Ammonium sulfate	740[b]	12,000	16.2	100
5. DEAE-cellulose I	570[b]	10,800	18.9	90
6. Sephadex G-75	3.2[c]	7,500	2300	62.5
7. Urea–LiCl	3.1[c]	3,900	1280	32.5
8. DEAE-cellulose II	0.38[c]	720	1880	6[e]

[a] From 1 lb of *E. coli* B cells. [b] Determined by biuret method. [c] Determined by OD_{280}. [d] Based on units. [e] 50% of the enzyme preparation was subjected to step 8. The values in the table are corrected to 100%.

2. *Properties of S Factor Protein. A. The nuclease content* of fraction 8 was tested by conversion of either [[^{3}H]T,[^{14}C]dC]-$d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ or [[^{3}H]T]-$d(T\text{-}G)_n$ to an acid-soluble form, and by conversion of covalently closed, circular PM2 DNA to the nickel form (see Methods). None of these assays gave a positive indication of DNase activity. A level of fraction 8 which reduced the clc-DNA content to less than 10% in the standard assay (10 μg/ml) produced no detectable acid solubilization of either $d(T\text{-}G)_n$ or $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ in 10 hr at 37°. This indicates that less than 1 nucleotide would have been removed nucleolytically for every 400 incorporated. The same level of S factor produced no detectable nicking of 1 OD_{260} PM2 DNA in 20 hr at 37°. This corresponds to less than 1 $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ molecule in the standard assay being endonucleolytically nicked for every 4×10^4 synthesized.

Polymers synthesized with fraction 7 or 8 factor present were of somewhat lower molecular weight than those made in its absence. Thus, three $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ samples made with S factor present had apparent single-strand molecular weights of 130,000 ± 6000, compared to 254,000 for polymers made without the factor, using Studier's (1965) equations. The average double-stranded molecular weights were 383,000 and 382,000, respectively.

B. *The heat lability* of fraction 8 was such that 50% of the activity was lost upon heating it to 47° for 7 min.

C. *Electrophoretic analysis* in sodium dodecyl sulfate containing 10% acrylamide gels showed primarily a single band, accounting for 90–95% of the protein in fraction 8 (Figure 4). A qualitatively similar pattern was seen with 30 μg of fraction 8 as with 4 μg. Comparison of the mobility of this band with known proteins, using the method of Shapiro *et al.* (1967), suggested a molecular weight of 11,500 for the protein in S factor.

D. *Stimulation of RNA polymerase* was studied at ratios of fraction 8 S factor to T4 DNA of from 1:1 to 20:1 (w/w). After 10, 20, 40, and 60 min at 37°, there was less than 10% difference between the amounts of RNA synthesized with and without S factor present. In all cases, an amount of RNA equivalent to 70–80% of the DNA template was synthesized in 60 min.

E. *S factor activity* was tested on two defined DNAs and *E. coli* DNA. Saturating levels of fraction 8 reduced the apparent clc-DNA content of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ only to about 5%, as measured by the fluorescence assay (Figure 2). This may have been a result of the template already having some clc structures, since the S factor is inactive on these, once formed (below). The defined DNA, $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$, is described in another paper (Coulter *et al.*, 1974), where it was

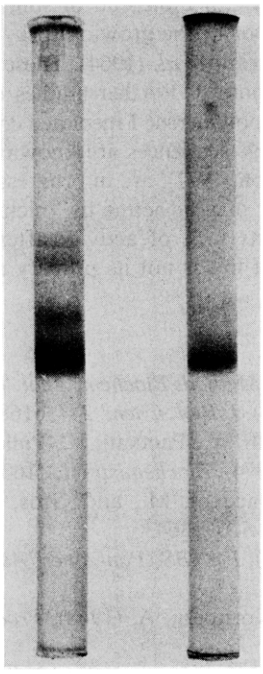

FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of S factor. Gels contained 10% acrylamide. Proteins were denatured by heating to 85° for 15 min in 10 mM dithiothreitol–0.33% sodium dodecyl sulfate. Electrophoresis was for 2 hr at 5 mA/gel. Gels were stained at 37° for 1.5 hr in Coomassie Brilliant Blue (Burgess, 1969), and destained electrophoretically in 7% acetic acid. Left, 10 μg of fraction 7 protein; right, 4 μg of fraction 8.

demonstrated that S factor also prevented the accumulation of clc sequences in this polymer, as determined by alkaline CsCl gradient centrifugation.

The inability of S factor to remove clc linkers during the course of subsequent DNA synthesis is illustrated in Figure 5. Addition of S factor at 1.5 or 2.5 hr after synthesis began did not remove the clc-DNA already formed by these times, but it did prevent some of the subsequent increase seen in its continued absence.

Fraction 8 S factor had no effect on isolated $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ of the clc type (Figure 6). Fraction 7, in the presence

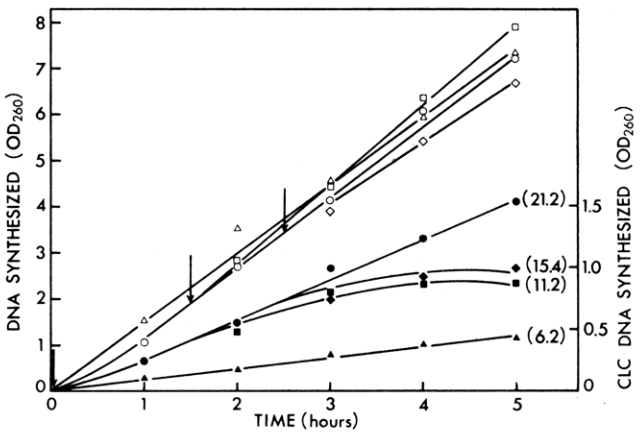

FIGURE 5: Synthesis of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ with clc sequences. DNA synthesis and clc-DNA production were determined with the fluorescence assay. Fraction 7 S factor (7.5 μg/ml) was added at 0 time (triangles), at 1.5 hr (squares), or at 2.5 hr (diamonds) of synthesis. DNase I was 25 ng/ml. Open symbols, total $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ synthesis; closed symbols, clc-DNA. Values in parentheses indicate per cent clc-DNA.

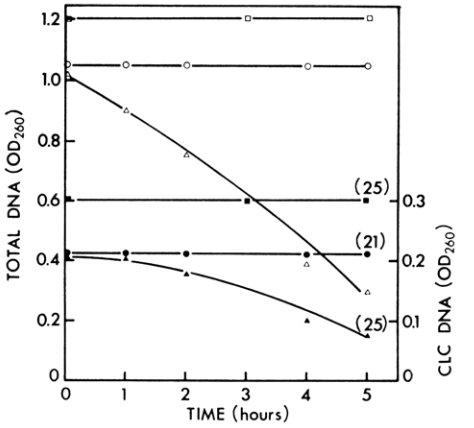

FIGURE 6: Effect of S factor on clc-DNA. Samples of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ containing 25% clc sequences were incubated at 37° with either 50 μg/ml of fraction 7 or fraction 8, in 30 mM potassium phosphate (pH 7.4), 2 mM dithiothreitol, 1 OD_{260} of DNA, 0.5 OD_{260} of yeast tRNA, and 12 mM $MgCl_2$ where indicated. Total DNA (open symbols) and clc-DNA (closed symbols) were determined by the fluorescence assay. The numbers in parentheses indicate the per cent clc-DNA at 5 hr: circles, fraction 7: triangles, fraction 7 + $MgCl_2$; squares, fraction 8 + $MgCl_2$.

of Mg^{2+}, contains sufficient nuclease to degrade the DNA under the conditions used, but the clc-DNA remained at 25% of the total.

These data indicate that S factor is not effective in removing clc linkers, once these have formed.

When *E. coli* DNA was used as the template for the DNA polymerase reaction, there were two effects of S factor. The reaction was inhibited, reaching a plateau at about onefold copying, and the clc-DNA level of the newly made DNA (corrected for the input template, which was 0% clc-DNA) was reduced to 67% (Figure 7). No further reduction was observed at 20 μg/ml of S factor. As expected, the absence of S factor led to the synthesis of extensive amounts of DNA, all of which was of the clc-DNA type.

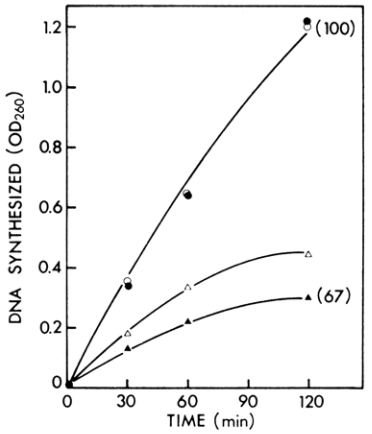

FIGURE 7: Effects of S factor on *E. coli* DNA synthesis by DNA polymerase. Standard assay conditions (see Methods) were used, except that the initial DNA template was 0.5 OD_{260}, and pancreatic DNase I was 50 ng/ml. Portions were removed from the synthetic reactions at the times indicated and analyzed for total (open symbols) and clc (closed symbols) DNA in the fluorescence assay with 20 mM K_3PO_4–0.2 mM EDTA buffer. Synthesis, at 37°, was either in the absence (circles) or presence (triangles) of 5 μg/ml of fraction 8 S factor. The contributions of the input DNA (0% clc) were subtracted. The numbers in parentheses indicate the per cent clc-DNA at 120 min.

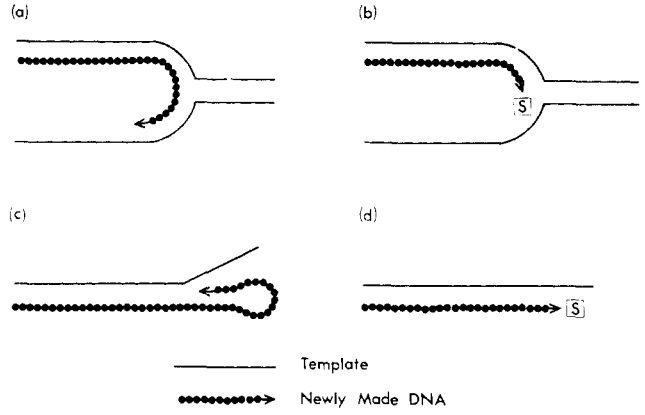

FIGURE 8: Models for the formation of clc-DNA by DNA polymerase, and the action of S factor. The structures in (a) and (c) represent two mechanisms for formation of clc-DNA; (b) and (d) indicate how S factor might block these two mechanisms. The growing, 3′(OH)-end of the new DNA is indicated by an arrow.

Discussion

This work describes a novel activity, that of blocking clc-DNA synthesis. This activity probably resides in a protein with molecular weight 11,500 in denatured form. The activity described, in its native form, behaves like a globular protein of about twice this molecular weight (26,000) on calibrated Sephadex G-75 columns.

The S factor is distinguishable from the *E. coli* single-stranded DNA binding protein described by Sigal *et al.* (1972), which has a molecular weight of 22,000 in sodium dodecyl sulfate. Furthermore, S factor does not produce hyperchromicity of T4 DNA under conditions similar to those shown by Sigal *et al.* (1972) to induce an apparent melting of T4 DNA (W. F. Flintoff *et al.*, unpublished data). S factor does not alter the kinetics of T4 DNA copying by RNA polymerase, making it apparently different from known proteins, of similarly low molecular weight, which stimulate this reaction (Cukier-Kahn *et al.*, 1972).

The purification of S factor presents several unusual features. The nature of the property assayed (lack of clc linkers) precludes a precise definition of its mode of action at present, and makes the definition of a unit of activity strictly operational. The ability of nonspecific nucleases to mimic S factor activity in the fluorescence assay dictates the use of the more difficult assay (alkaline CsCl gradient centrifugation) at earlier stages of purity (Coulter *et al.*, 1974). The greatest difficulty is in the apparent association between S factor and a putative oligo [d(A-T)]. Separation was only possible after the protein was denatured by the urea–LiCl solvent.

The purified S factor is effective during the synthesis of two defined DNAs, $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$. The effects observed on the copying of *E. coli* DNA, inhibition of clc formation and inhibition of extensive synthesis, are consistent with S factor's inhibiting the turning around of DNA polymerase at either the end of single-stranded DNA (Hayes *et al.*, 1971) or at the growing fork, as proposed, for example, by Schildkraut *et al.* (1964). These modes of action are described in Figure 8. Further studies on the action of purified S factor on polymerase I mediated copying of circular PM2 DNA, where clc sequences are known to occur (Masamune and Richardson, 1971), are in progress.

Although S factor may be acting by "nicking" clc-DNA at the growing fork, its lack of activity after clc linkers are formed suggests that this is not its primary mode of action.

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