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In Vitro Synthesis and Detection of Deoxyribonucleic Acids with Covalently Linked Complementary Sequences†

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ABSTRACT: An activity has been demonstrated which blocks the accumulation of DNA with covalently linked, complementary (clc) sequences during the copying of defined DNAs with DNA polymerase I of *Escherichia coli*. The factor is necessary for the synthesis of both d(T-G)$_n$·d(C-A)$_n$ and d(T-T-G)$_n$·d(C-A-A)$_n$ free of clc sequences. A modification of the ethidium fluorescence method for measuring clc sequences is described. This facilitates measurement of clc structures in natural polynucleotides without interference by nonspecific, intrastrand associations. The defined polymer d(T-T-G)$_n$·d(C-A-A)$_n$ has been prepared with separable strands. Defined DNAs of the type d(purine)$_n$·d(pyrimidine)$_n$ do not give rise to clc-DNA, even in the absence of the above factor, in our experience. Since the complementary strands of d(T-C)$_n$·d(G-A)$_n$ have the same buoyant densities in alkaline CsCl, an independent method was developed to separate them physically, and thereby prove that the polymer was of the non-clc type. In this method, the polymer was treated with a water-soluble carbodiimide under mild conditions, to derivatize T and G residues. The strands were separated by neutral CsCl equilibrium centrifugation, and dederivatized by mild alkali to regenerate the original strands.

Naturally occurring DNAs generally consist of complementary strands which may be topologically, but not covalently, linked. Several exceptions exist: newly synthesized phage T7 DNA (Barzilai and Thomas, 1970) and λ-DNA (Ihler and Kawai, 1971) appear to contain covalent linkers between complementary strands, at least under some conditions. Similarly, electron micrographs of replicating colicin E1 DNA (Fuke and Inselburg, 1972) can be interpreted to indicate covalent continuity between the complementary daughter strands at the growing fork. During the *in vitro* copying of natural (Schildkraut *et al.*, 1964) and defined (Paetkau, 1969; Harwood and Wells, 1970) DNAs by DNA polymerases, covalent linkers appear between complementary sequences (clc sequences[1]). Clc sequences also arise when DNA becomes cross-linked by agents such as mitomycin C. A technique for detecting such structures has been developed (Morgan and Paetkau, 1972), based on the enhanced fluorescence of ethidium bromide when it is bound to bihelical DNA (LePecq and Paoletti, 1967). In the present work, this technique has been modified to prevent the nonspecific, relatively unstable, bihelical structures occurring in heat-denatured, naturally occurring DNAs. Several other procedures are described which permit either separation of the complementary strands of synthetic DNAs of defined sequence, or detection of clc structures when these exist. Conditions for enzymatically synthesizing defined polymers free of clc sequences are described.

Experimental Section

Materials. Chemicals. CsCl was obtained from Pierce Chemical Co. or Schwarz/Mann and *N*-cyclohexyl-*N'*-β-(4-methylmorpholium)ethylcarbodiimide *p*-toluenesulfonate (hereafter referred to as the carbodiimide) from Aldrich Chemical Co. Technical grade Cs_2SO_4 was obtained from American Potash and Chemical Corporation and purified by recrystallization from hot water. BrdUTP was prepared by a modification (D. E. Pulleyblank and A. R. Morgan, unpublished) of the procedure of Inman and Baldwin (1964). Other materials were indicated earlier (Morgan *et al.*, 1974).

Enzymes. Endonuclease I was purified from *E. coli* by the method of De Waard and Lehman (1966). A unit of endonuclease I is defined as causing a hyperchromicity of 0.001

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[1] Abbreviations used are: clc-DNA, DNA with covalently linked, complementary sequences; BrdUTP, deoxybromuridine 5'-triphosphate.

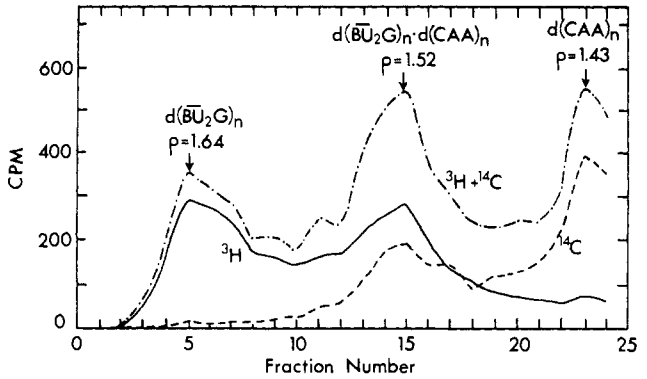

FIGURE 1: Preparative alkaline Cs_2SO_4 equilibrium centrifugation of d(BrU-BrU-G)$_n$·d(C-A-A)$_n$. The centrifugation was performed in a Ti 50 rotor at 40,000 rpm for 65 hr at 20°. The Cl_3CCOOH insoluble counts were determined in aliquots from the gradient following fractionation. Corrections were made for overlap of the single-isotope channels. Densities were determined refractometrically. Fractions 4–7 and 22–24 inclusive were pooled separately and neutralized, dialyzed, and concentrated by vacuum dialysis *vs.* 0.005 M Tris-Cl (pH 8)–0.1 mM EDTA. The fractions pooled were assumed to consist of d([^{3}H]BrU-BrU-G)$_n$ and d([^{14}C]C-A-A)$_n$, respectively, on the basis of their labeling. The middle portion of the gradient labeled with both ^{3}H and ^{14}C was assumed to consist of d([^{3}H]BrU-BrU-G)$_n$ covalently linked to d([^{14}C]C-A-A)$_n$.

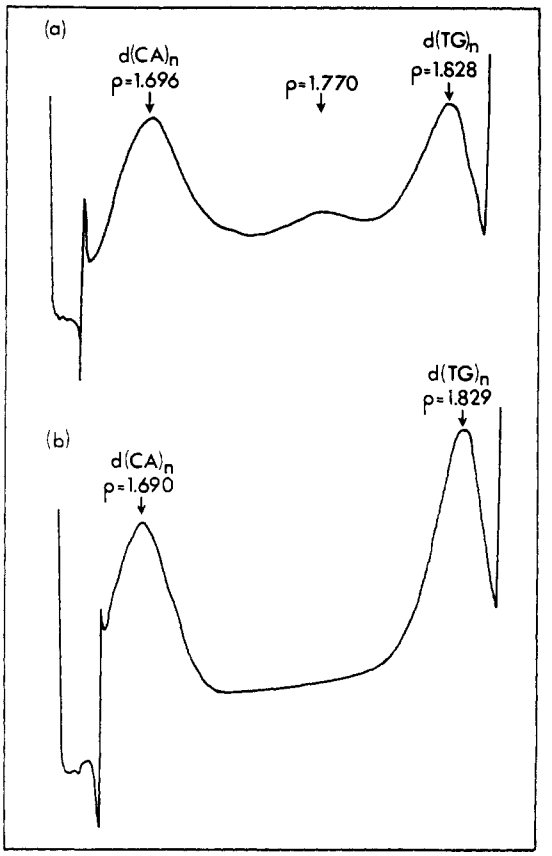

FIGURE 2: Analytical CsCl gradient ultracentrifugation of d(T-G)$_n$·d(C-A)$_n$. Polymers were synthesized either with (b) or without (a) fraction DIII, isolated by Agarose chromatography and centrifuged to equilibrium in alkaline CsCl gradients. Conditions of centrifugation were: 48,000 rpm, at 20°, for 22 hr. Densities were determined by the isoconcentration method (Vinograd, 1963). Samples are further characterized in Table I.

OD_{260} unit/min at 25°. RNA polymerase was prepared to fraction 3 by the method of Burgess (1969) and further purified as for fractions III–VI by the method of Paetkau and Coy (1972), omitting the 0.5 M Agarose step and substituting DEAE Sephadex for QAE-Sephadex. *E. coli* DNA polymerase I and fraction DIII were obtained as indicated earlier (Morgan *et al.*, 1974).

DIII was added to DNA polymerase reactions synthesizing d(T-G)$_n$·d(C-A)$_n$ or d(T-T-G)$_n$·d(C-A-A)$_n$ to prevent the accumulation of clc sequences. When DIII was heated in boiling water for 10 min, about half this activity was lost. Heated DIII still retained a low level of apparent endonuclease I activity. To block endonuclease I activity in DIII and heated DIII, tRNA (0.5 OD_{260}) was added to these DNA polymerase reactions (Lehman *et al.*, 1962).

A protein factor S (for separability of strands), having similar activity to DIII, has been purified (Flintoff and Paetkau, 1974) from DNA polymerase fraction 4 (Jovin *et al.*, 1969). This protein was used for the synthesis of d(T-T-G)$_n$·d(C-A-A)$_n$.

Methods. DNA Synthesis. (a) General. The basic procedure for enzymatic synthesis of DNAs *in vitro* was as described in Morgan *et al.* (1974). Unless otherwise indicated, dNTPs were present at a total concentration of 5.2 mM and in the molar ratios at which they occur in the given template. The extent of DNA synthesis was determined either by the conversion of radioactive dNTPs into a Cl_3CCOOH insoluble form, or by the formation of a fluorescent complex when ethidium bromide was added (Morgan and Paetkau, 1972; Morgan *et al.*, 1974). Clc sequences were determined in one of two buffer systems. For synthetic defined polymers, DNA was diluted to 0.02 OD_{260} in 2 mM Tris-Cl–0.2 mM EDTA, measured pH 8.4. For natural DNAs the preferred buffer was 20 mM K_3PO_4–0.2 mM EDTA, measured pH about 12. The 20 mM K_3PO_4–0.2 mM EDTA buffer destroys the relatively nonspecific structures observed with denatured natural DNAs (Morgan and Paetkau, 1972) but does not affect specific bihelical DNA (see Figure 8). To measure total bihelical DNA content, ethidium bromide was added directly to these diluted samples to a final concentration of 0.5 μg/ml, and the fluorescence was compared to standard samples of DNA, thereby giving the concentration of synthesized DNA as OD_{260}. To determine clc structures, parallel samples were placed in boiling water for 5 min and cooled on ice before ethidium bromide was added. Under these conditions, only clc structures give fluorescence (Morgan and Paetkau, 1972). The Agarose chromatography method (Morgan *et al.*, 1974) was used to isolate polymers.

(b) *d(T-T-G)$_n$·d(C-A-A)$_n$ Synthesis.* The defined polymer d(T-T-G)$_n$·d(C-A-A)$_n$ was enzymatically copied from defined, synthetic oligonucleotides in the laboratory of H. G. Khorana (Wells *et al.*, 1967). The segregation of ionizable bases in this polymer and experience with other defined polymers suggested that the d(T-T-G)$_n$ and d(C-A-A-)$_n$ strands should separate in an alkaline CsCl gradient. The starting material available to us, however, formed a single band in an alkaline CsCl gradient (R. D. Wells, personal communication). The product made from this with DNA polymerase I, polymer A, contained 30% clc sequences. In order to exaggerate the difference in density between the d(T-T-G)$_n$ and d(C-A-A)$_n$ strands and thus obtain the separate strands, polymer A was copied in the presence of BrdUTP (1.8 mM) with a trace of [^{3}H]TTP (0.2 μM) and [^{14}C]dCTP. The product was isolated by Agarose chromatography and then subjected to preparative alkaline Cs_2SO_4 equilibrium centrifugation as described in Figure 1. The individual strands, free of clc material, were pooled as

TABLE I: Determination of Clc Sequences in $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ by Various Techniques.

Condition of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ Synthesis	Fluorescence Clc Assay (%)	Nearest Neighbor dNpdA (%)			CsCl Gradient
		N = A	N = G	N = T	
1. Polymerase alone	25	3.1%	7.8% (Figure 3a)	3.7%	Figure 2a
2. Polymerase + DIII	4	<0.6%	<1.0% (Figure 3b)	1.9%	Figure 2b

indicated and mixed. The strands were annealed at low ionic strength (0.005 M Tris-Cl (pH 8)–0.1 mM EDTA) by heating in boiling water and slowly cooling (45 min) to room temperature. The annealed product was used in the usual DNA polymerase reaction with the addition of 0.5 ng/ml of pancreatic DNase I to stimulate the reaction, 0.5 OD_{260} yeast tRNA to inhibit endonuclease I, and 1:20 v/v heated DIII. The product (polymer B) was demonstrated to be $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$ without clc sequences by (i) transcription with RNA polymerase, in which incorporation of CTP depended on ATP and the ratio of C to A incorporated was 1:2; (ii) a T_m of 69° in $^1/_{10}$ ssc in agreement with the literature value (Wells *et al.*, 1970); and (iii) analytical alkaline CsCl gradient centrifugation, which showed two separable bands. Polymer B was used as template for subsequent syntheses.

Chemical Modification of Thymine and Guanine Residues in Polynucleates. The carbodiimide was used to modify $d(T\text{-}C)_n \cdot d(G\text{-}A)_n$ essentially by the procedure of Gilham (Lee *et al.*, 1965; Ho and Gilham, 1967). The polymer (2.5 μmol of total nucleotide) was dissolved in 0.5 ml of 0.06 M sodium carbonate, 0.5 ml of 20% w/v of the carbodiimide (bromide form) in ethanol was added, and the pH adjusted to 9.5 with NaOH. The reaction was performed at 30° and monitored at 296 mμ. Derivatized DNA was recovered by chromatography on a 0.5 M Agarose column in 0.05 M potassium phosphate (pH 6.5)–0.1 mM EDTA. CsCl was added for preparative or analytical ultracentrifugation (Figure 7). The recovered, separated, strands were dederivatized at pH 12.5. Identification of the strands was based on: (i) the more rapid dederivatization of the $d(G\text{-}A)_n$ adduct (Ho and Gilham, 1967), (ii) selective transcription of the $d(T\text{-}C)_n$ strand to $r(G\text{-}A)_n$ by RNA polymerase, and (iii) spectral properties. The individual strands showed no fluorescence with ethidium bromide, but when mixed and annealed, re-formed fluorescence-enhancing structures.

Other methods were as described elsewhere (Morgan *et al.*, 1974).

Results

Effect of Fraction DIII on Clc Sequences in $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. The separable-stranded, defined polymer $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was copied *in vitro* with DNA polymerase, under the conditions described in Methods. One sample was prepared with only polymerase and pancreatic DNase I (0.05 μg/ml) present, the latter to stimulate synthesis. The second sample was prepared with DIII (1:50 v/v) and 0.5 OD_{260} tRNA present in addition to polymerase and DNase I. The two samples were analyzed by fluorescence (Table I) and gave 25% clc and 4%, respectively. They were isolated and examined by analytical

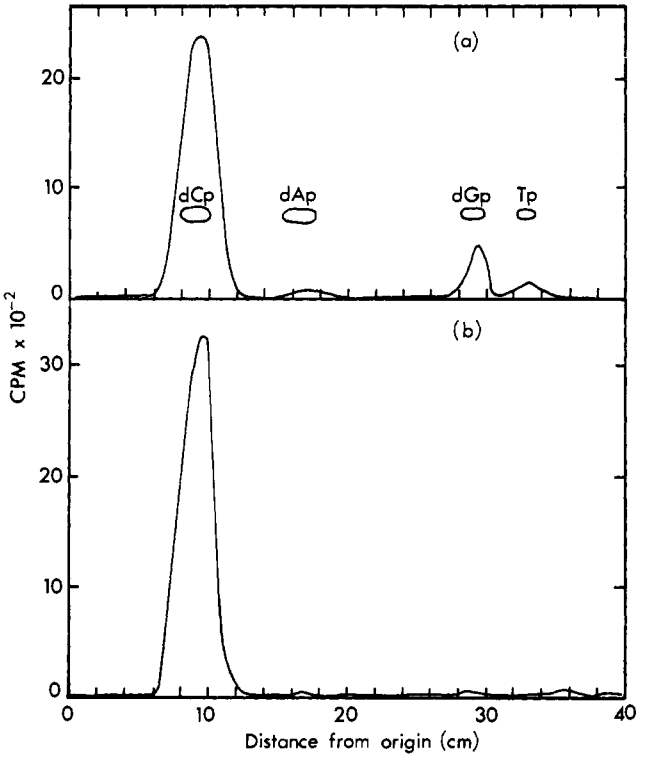

FIGURE 3: Nearest neighbor analysis of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ by high-voltage electrophoresis. Samples were synthesized with [α-^{32}P]-dATP with (b) and without (a) fraction DIII. They were then analyzed for nearest neighbors as described in the Methods. Samples are further characterized in Table I.

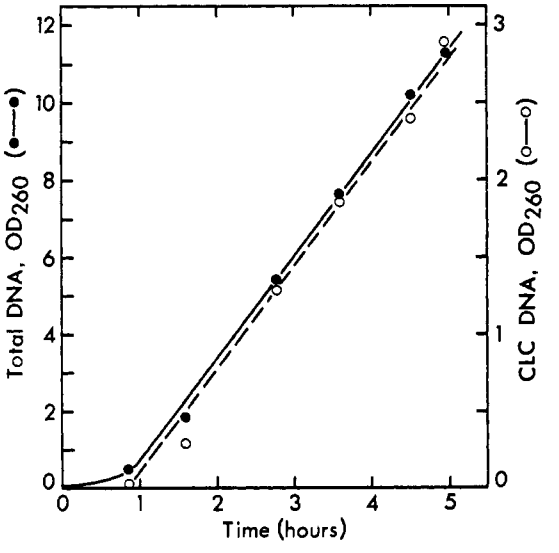

FIGURE 4: Kinetics of clc synthesis. $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ was synthesized in the absence of DIII. Total DNA synthesis and the amount which contained covalently linked complementary sequences (clc) were measured with the fluorescence technique (see Methods).

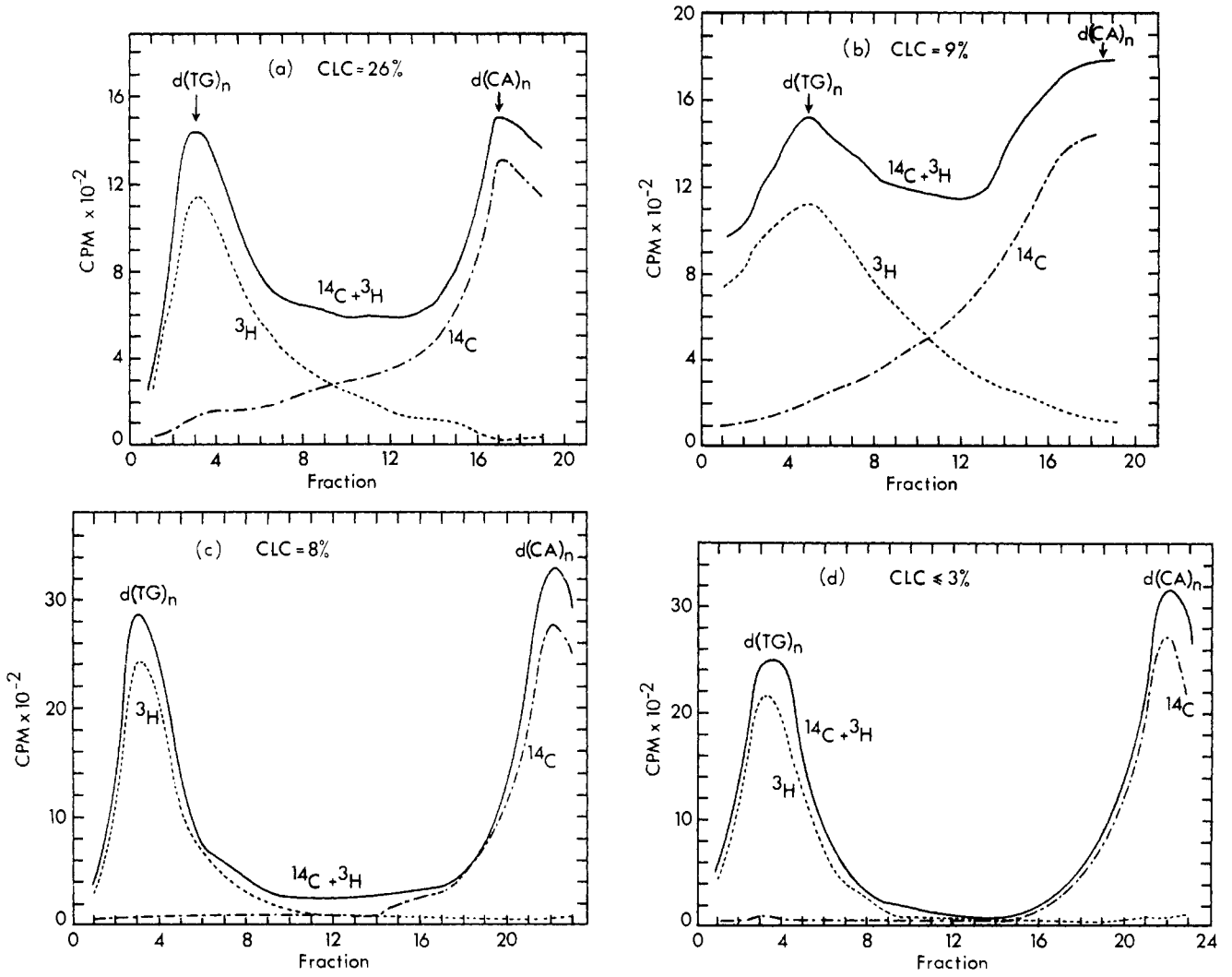

FIGURE 5: $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ synthesized from a separable-stranded template in the presence of DNA polymerase alone (a), or in the presence of 0.35 unit/ml of endo I (b), 1:50 v/v DIII (c), or 1:20 v/v heated DIII (d). Clc determinations were performed by the ethidium bromide method. DNA samples synthesized under the conditions indicated (30–60-fold copying of the input template) were prepared with [^{3}H]TTP and [^{14}C]dCTP. Aliquots were removed and added to a solution of alkaline CsCl such that the final density would be 1.760 g/cm^3. Preparative gradient centrifugation was carried out in a Ti 50 rotor at 38,000 rpm for 65 hr at 20°. The gradients were fractionated, aliquots removed, and Cl_3COOH insoluble counts determined. The efficiency of counting for the isotopes was slightly higher in the "^{14}C + ^{3}H" channel than in the single-isotope channels. Corrections were made for channel overlap. Densities of individual fractions were determined refractometrically, and the positions of $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$ assigned to their literature values (Wells and Blair, 1967).

alkaline CsCl equilibrium centrifugation (Figure 2) and nearest neighbor analysis (Figure 3). For nearest neighbor analysis, the polymers were further copied, in the presence of [α-^{32}P]-dATP, and either with or without DIII. The CsCl results for the first sample indicate material with a buoyant density between that of $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$, as observed before (Paetkau, 1969; Harwood and Wells, 1970). This material consists of poly[d(T-G)] linked in an alkali-stable form to poly[d(C-A)]. The level of clc material observed in each sample is consistent with the results of centrifugation. The 4% clc seen with polymer made in the presence of DIII is near the lower level of detection, but may be significant. Its possible origin is considered in the Discussion. Finally, the clc polymer contains a significant level of "wrong" nearest neighbors to dA, compared to the polymer made with DIII present. This is consistent with the occurrence of phosphodiester linkers between the complementary sequences.

Kinetics of Synthesis of Clc Sequences in $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$. When $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ with separable strands is used as template, and DIII is not present, clc sequences appear after about 1 hr. This is illustrated by Figure 4. Clc sequences appeared after about twofold copying of the template had occurred (initial template was $OD_{260} = 0.2$), and increased rapidly to 25% of the total DNA. This polymer typically gave 20–25% clc by the fluorescence assay when DIII was omitted from the polymerase reaction mixture.

Analysis of $d([^3H]T\text{-}G)_n \cdot d([^{14}C]C\text{-}A)_n$ Made under Various Conditions. The data shown in Figure 5a extend the information contained in Figure 2a by showing the distribution of clc containing $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ in which the sequences were differentially labeled with [^{3}H]T and [^{14}C]dC. As expected, the tritium and ^{14}C labels extended into regions of the gradient removed from the appropriate densities of $d(T\text{-}G)_n$ and $d(C\text{-}A)_n$. Experiments indicated that endonucleases could, at sufficiently high concentrations, decrease the apparent clc content of polymers as measured by fluorescence. Therefore, since DIII contains endonuclease I, a sample prepared with endo I present was also examined. Under the conditions used, the product registered only 9% clc in the fluorescence assay. The product was obviously highly degraded, and probably contained a high level of actual clc structures (Figure 5b). In sharp contrast, DIII (Figure 5c) reduced the clc content and led to

TABLE II: Effect of Template State on the Type of $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ Synthesized in the Presence of DIII.[a]

Expt	Template and Pretreatment	Type of Product Formed	Net-Fold Synthesis
1	Double-stranded mol wt 175,000	Separable-stranded material (no clc)	65×
2	Same as 1, heat denatured and rapidly cooled	Some intermediate density material (clc)	70×
3	Template used in 1 degraded to double-stranded mol wt 21,000 before synthesis	Separable-stranded material; <8% clc sequences	85×
4	Intermediate density template	Intermediate density material (clc)	70×

[a] DNA polymerase reactions were carried out under standard conditions in the presence of 30 μg/ml of DIII. Net-fold synthesis was determined as the amount of polymer recovered from Agarose chromatography (see Methods) compared to the input template. For expt 2, the template was heated in boiling water for 5 min, then quick-cooled. For expt 3, the template from expt 1 was degraded with 0.7 unit of endo I for 30 min at 37° and isolated by Agarose chromatography. Molecular weights were determined by the method of Studier (1965).

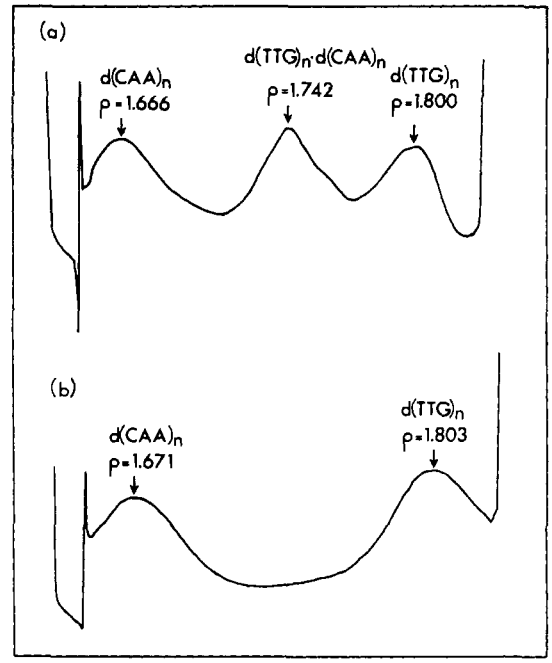

FIGURE 6: Analytical alkaline CsCl gradient ultracentrifugation of $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$. Polymers were prepared either with (b) or without (a) purified S factor, isolated by Agarose chromatography and centrifuged to equilibrium in alkaline CsCl gradients. Conditions of centrifugation were 48,000 rpm at 20° for 45 hr. Densities were determined by the isoconcentration method. The segregation of ionizable bases permits the assignment of $d(T\text{-}T\text{-}G)_n$ and $d(C\text{-}A\text{-}A)_n$ to the highest and lowest density bands, respectively.

the synthesis of sharply separated strands. Heated DIII was not effective at the same dilution, but at 1:20 v/v (Figure 5d) it was even more effective than DIII. There was no detectable overlap of $d(T\text{-}G)_n$ (^3H label) and $d(C\text{-}A)_n$ (^{14}C label). The systems in Figures 5c and 5d contained tRNA (0.5 OD_{260}) to block endonuclease I. In separate experiments, no concentration of endonuclease I, with or without tRNA present, mimicked the activity of DIII in blocking the accumulation of clc sequences.

In the presence of tRNA, both DIII and heated DIII led to a higher molecular weight product than polymerase by itself (with or without tRNA). The product shown in Figure 5a, for example, had a molecular weight of 162,000 in alkali and 339,000 at neutral pH, whereas the material in Figure 5d gave values of 265,000 and 455,000, respectively, for these parameters. This effect has been reproducible.

Effect of Template Pretreatment on Clc Synthesis. Degrading the template with endonuclease I did not affect the nature of the product DNA.

Heating and rapidly cooling $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ produce highly branched structures (unpublished electron micrographs). This material was a template for clc sequence formation, even with DIII present (Table II).

Effect of S Factor on Clc Sequences in $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$. Polymer B (see Methods) was used as a template to determine the effect of S factor on synthesis of $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$. Polymer C was the product of the reaction when 0.1 μg/ml DNase I only was added to the DNA polymerase system. Polymer D was prepared from polymer B in the presence of S factor and 0.5 OD_{260} of tRNA in addition to DNase I. Both polymers were isolated and examined by analytical alkaline CsCl equilibrium centrifugation (Figure 6). Polymer D showed two separated peaks, of densities 1.671 g/cm^3 and 1.803 g/cm^3, and no material in the middle (*i.e.*, no clc material). Polymer C, made in the absence of S, showed in addition a band of intermediate density. The T_m of both polymers agreed with the literature value of $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$ (Wells *et al.*, 1970).

Analysis of Polypyrimidine·Polypurine DNA for Clc Sequences. Polymers of the type $d(polypyrimidine)_n \cdot d(polypurine)_n$ have not been observed to contain clc sequences by the fluorescence assay, even when synthesized in the absence of DIII. The separation of the strands of $d(T\text{-}C\text{-}C)_n \cdot d(G\text{-}G\text{-}A)_n$ in an alkaline CsCl gradient supports this observation (Morgan *et al.*, 1974). In the case of $d(T\text{-}C)_n \cdot d(G\text{-}A)_n$ the complementary strands do not separate sufficiently to permit their analysis by alkaline CsCl banding. However, when the polymer was derivatized with the carbodiimide and centrifuged to equilibrium in neutral CsCl, the two derivatized strands separated as shown in Figure 7. Again there was no evidence for intermediate density material. In a parallel preparative gradient, the strands were recovered, dederivatized, and identified as described in the Methods.

E. coli DNA In Vitro. The product of this natural DNA copied by DNA polymerase *in vitro* was analyzed by the fluorescence technique. The newly made polymer was 100% clc (Figure 8), whereas the input template, in the 20 mM K_3PO_4–0.2 mM EDTA buffer system, was 0%. This agrees with the observation of Schildkraut *et al.* (1964) and gives a quantitative measure of clc structure formation.

Discussion

The synthesis of spontaneously renaturing, or clc sequence, DNA by DNA polymerase I *in vitro* was first demonstrated by Schildkraut *et al.* (1964). Subsequent reports demonstrated that, in copying the defined template $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$, both *E. coli* polymerase I (Paetkau, 1969) and *Micrococcus luteus*

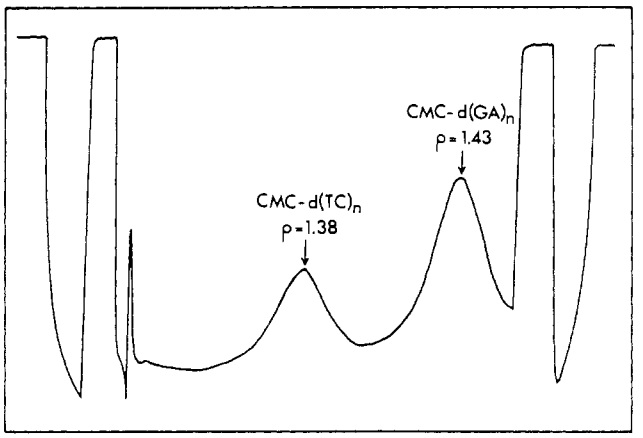

FIGURE 7: Analytical ultracentrifugation of carbodiimide-derivatized d(T-C)$_n$·d(G-A)$_n$. Derivatization was performed according to Methods. Conditions of centrifugation in neutral CsCl were 48,000 rpm at 20° for 24 hr. Densities were determined from a parallel, preparative gradient experiment and peak assignments were made on the basis of data described in Methods.

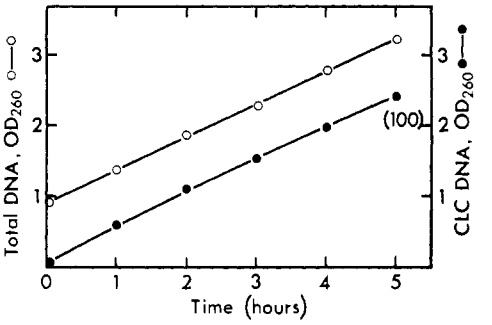

FIGURE 8: *E. coli* DNA synthesis. *E. coli* DNA (1.0 OD_{260}) was used as a template for the polymerase reaction, which was performed under standard conditions except that the phosphate buffer (pH 6.7) was 0.067 M. Samples were analyzed either directly, or after heating and cooling, in 20 mM K_3PO_4–0.2 mM EDTA (pH 12) buffer. The number 100 represents the percentage of the newly synthesized DNA which is clc.

polymerase (Harwood and Wells, 1970) would produce some clc structures. Recent work indicates that both calf thymus DNA polymerase (Hayes *et al.*, 1971) and T4 DNA polymerase (Englund, 1971) can synthesize clc structures, although their mechanisms may differ. A reasonable model for the formation of such structures would involve turning around of the polymerase at the growing fork, or strand switching. That this does in fact occur with *E. coli* acting on a circular, natural DNA template *in vitro* is demonstrated by the work of Masumune and Richardson (1971).

The importance of clc sequences as replication intermediates *in vivo* is difficult to assess. Since nascent DNA may be part of complex, sensitive structures, the apparent lack of clc sequences found by Burger (1971) in *Bacillus subtilis* is less compelling than the evidence for their existence in T7 (Barzilai and Thomas, 1970) and λ (Ihler and Kawai, 1971). In both these bacteriophage DNAs the clc sequences occurred in DNA molecules up to unit length and longer. They do not, therefore, fulfill the expectation for nascent DNA, on the basis of the discontinuous DNA synthesis model of Okazaki *et al.* (1968). Furthermore, we have not found a clc DNA in T7 at anywhere near the levels found by Barzilai and Thomas (L. Langman and V. Paetkau, unpublished). Other work suggesting that replicating DNA contains clc sequences is described by Fuke and Inselberg (1972), where electron micrographs of partly replicated "rolling circle" DNA molecules are consistent with a covalent linker between daughter strands at the growing fork.

The relation of the present studies to DNA replication *in vivo* is still unclear. Polymerase I does appear to be directly involved in the replication of colicin E1 *in vivo* (Goebel, 1972). Furthermore, polymerase III, which is required for *E. coli* replication (Gefter *et al.*, 1971; Nüsslein *et al.*, 1971), appears to catalyze a similar synthetic reaction to that of polymerase I (Kornberg and Gefter, 1972). This suggests that under some conditions clc linkers may be metastable replication intermediates. S factor activity could be responsible for preventing the accumulation of clc linkers *in vivo*.

The present work demonstrates the existence of an activity which blocks the formation of clc sequences *in vitro*, at least with synthetic templates. These polymers may be copied in a complex fashion, with chain elongation occurring both by a strand displacement mechanism and by a slippage mechanism. Only the former would likely lead to strand switching. Thus it may be significant that the repeating trinucleotide polymer d(T-T-G)$_n$·d(C-A-A)$_n$, which would be less likely to slip than the repeating dinucleotide polymer d(T-G)$_n$·d(C-A)$_n$, also gives a higher percentage of clc sequences in the absence of S factor. Preliminary experiments on the copying of *E. coli* DNA *in vitro* indicate that the purified S factor partly inhibits synthesis, and partly blocks clc accumulation (Flintoff and Paetkau, 1974). Further studies, using natural DNAs with defined physical structures, are in progress. A reasonable hypothesis for the mode of action of S factor is that it binds to the exposed single-stranded DNA template thrown out during the polymerase reaction and prevents the polymerase from switching strands and forming clc structures. Premature strand switching could also produce a fraction of lower molecular weight DNA, as observed.

The correlation of results obtained by the fluorescence, ultracentrifugal, and nearest neighbor analyses lends confidence to the use of the most facile of these (the fluorescence assay) to the purification of the S factor. Endonuclease activity gives an apparent decrease in clc-DNA (fluorescence assay) by removing material from clc sequences (Figure 5). At the level of DIII or fraction 4, it is necessary to block endonuclease I with tRNA to make the fluorescence assay a reliable measure of clc-DNA synthesis. The purified S factor itself, however, contains no nuclease activity (Flintoff and Paetkau, 1974). The low level of clc sequences observed with less than saturating DIII when the fluorescence, but not the ultracentrifuge assay, is used suggests a real difference between these methods. The difference may arise from low molecular weight, clc sequences, which register fluorescence but do not form bands in the ultracentrifuge. Such sequences must be very low in molecular weight, for a polymer of alkaline molecular weight 14,000 produces discrete bands (see Figure 5b).

Our present information indicates that the S factor activity in DIII is partially heat-stable, trypsin sensitive, and not mimicked by endonuclease I with or without tRNA present, protamine sulfate, or varying ionic stength. The heat stability of the activity in DIII may be due to the presence of protamine sulfate used in its isolation. Purification of the activity in DIII has proven difficult because of protamine sulfate and other contaminants, and we have therefore purified a similar activity from fraction 4 DNA polymerase. This purified S factor differs from the single-strand binding protein of *E. coli* (Sigal *et al.*, 1972) in its molecular weight and other properties (Flintoff and Paetkau, 1974).

The observation that polypyrimidine·polypurine DNAs

never contain clc sequences appears to be a peculiar property in the synthesis of these DNAs. The carbodiimide derivatization method used for these polymers should be generally applicable to DNAs in which the strands, whether complementary or from different genetic regions, have differing T and G contents, since it is specific for these bases. The isolated, dederivatized strands obtained from $d(T\text{-}C)_n \cdot d(G\text{-}A)_n$ after separation by this method were not grossly altered in size, and served as templates for RNA polymerase.

The presence of an activity which blocks clc-DNA synthesis in $d(T\text{-}G)_n \cdot d(C\text{-}A)_n$ and $d(T\text{-}T\text{-}G)_n \cdot d(C\text{-}A\text{-}A)_n$ solves a practical problem, that of preparing these DNAs with separable strands. These polymers can thus be reproducibly copied without the frequent occurrence of clc linkers.

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