Periodic Cleavage of Poly(dA) by Oligothymidylates Covalently Linked to the 1,10-Phenanthroline–Copper Complex†

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ABSTRACT: 1,10-Phenanthroline (OP) was covalently attached to the 3′-terminus of two oligothymidylates via different linkers [abbreviated as T_8-(OP) and T_6-(OP)]. In the presence of Cu^{2+} and 3-mercaptopropionic acid (MPA), these reagents induce a hybridization-dependent cleavage of poly(dA) and of a 27 nucleotide long oligodeoxynucleotide containing an A_8 sequence. The principal cleavage sites on the 27-mer span four residues located near the 3′-terminal phosphate group of T_8-(OP). When poly(dA) was degraded by T_6-(OP) and T_8-(OP), a series of bands were obtained corresponding to a repeat unit of six and eight nucleotides, respectively. This periodicity reflects the cooperative binding of oligothymidylate–OP to the polynucleotide matrix and the localized nicking sites.

Synthesis of new families of substances which could be used to inactivate selectively a predetermined region in a gene or messenger RNA has been the subject of active research during the past years. It has been reported that synthetic oligonucleotides could inhibit transcription (Hélène et al., 1985) and translation (Zamecnik et al., 1985; Knorre & Vlassov, 1985; Blake et al., 1985; Toulmé et al., 1986; Cazenave et al., 1986) both in vitro and in vivo. Oligonucleotides form reversible complexes with their target sequence and do not completely inhibit biological processes. So arose the idea of associating an active reagent to the oligonucleotide in order to induce irreversible reactions in the target sequence [see Knorre and Vlassov (1985) for a review]. Such composite molecules include compounds that interact strongly and selectively with a target nucleic acid sequence and reactive groups that can cleave the phosphodiester backbone of nucleic acids. Oligonucleotides of defined sequence coupled to active groups such as EDTA[1]–Fe(II) (Boutorin et al., 1984; Chu & Orgel, 1985; Dreyer & Dervan, 1985; Boidot-Forget et al., 1986), porphyrin–Fe(III) (Le Doan et al., 1986), and 1,10-phenanthroline–Cu(I) (Chen & Sigman, 1986) have been shown to be efficient for the cleavage of complementary nucleic acid sequences in the presence of a reducing agent in aerated solutions.

In this paper we present results on the cleavage of poly(dA) and a 27-mer containing the target sequence by complementary oligothymidylates substituted with 1,10-phenanthroline at the 3′-end. We show that these molecules induce a periodic cleavage of the polynucleotide chain.

Materials and Methods

Poly(dA) and poly(dT) were purchased from P-L Biochemicals. ^{32}P-5′-labeled polynucleotides resulted from dephosphorylation of the corresponding polynucleotides with bacterial alkaline phosphatase (BRL) and phosphorylation with [γ-^{32}P]dATP and T4 polynucleotide kinase (Amersham). ^{32}P-3′-labeling was carried out with [α-^{32}P]ddATP and nucleotidyl terminal transferase (Amersham). The 27-mer was synthesized on an Applied Biosystems synthesizer and purified by polyacrylamide gel electrophoresis followed by reverse-phase chromatography. End labeling (5′-side) was achieved by polynucleotide kinase with γ-^{32}P-radiolabeled ATP (Amersham).

Synthesis of Phenanthroline-Substituted Oligothymidylates. Two oligothymidylates [T_6-(OP) and T_8-(OP)] containing OP attached at their 3′-end were synthesized according to Scheme I.

(*A*) *Synthesis of ω-Hydroxy–Linker–OP.* One equivalent of compound **1** (a kind gift from Dr. D. Sigman) was reacted with 3 equiv of 3-aminopropanol in acetonitrile at room temperature for 1 h. After evaporation of the solvent under vacuum, the product was purified on a Lichroprop RP-18 column (Merck) with a linear gradient of H_2O–MeOH. Compound **2** was eluted with 35% methanol.

(*B*) *Synthesis of T_6-(OP).* A solution of the fully protected hexathymidylate [DMTr(Tp^o)$_6$ (1.2 equiv)] and compound **2** was treated with mesitylenesulfonyl tetrazolide (MSTe) (3 equiv) for 45 min at room temperature. The excess of MSTe was destroyed by addition of water. The protected oligomer **4** was chloroform-extracted and purified by silica 60 gel chromatography with mixtures of CH_2Cl_2 and MeOH [92:8, 86:14, and 80:2 (v/v)]. The fraction containing the oligomer **4** was dried by coevaporation with pyridine and treated for 24 h at room temperature with an equimolar solution of benzohydroxamic acid (BHA) and 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) in pyridine (10 equiv of BHA–DBU for one phosphotriester group). Then T_6-(OP) was purified by HPLC.

(*C*) *Synthesis of T_8-(OP).* The attachment of a thiophosphate group at the 3′-end of oligothymidylates has been described elsewhere (Praseuth et al., 1987). A mixture of (Tp)$_7$T(ps) (1 equiv) and 5-(iodoacetamido)-1,10-

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[1] Abbreviations: OP, 1,10-phenanthroline; EDTA, ethylenediaminetetraacetic acid; MPA, 3-mercaptopropionic acid; Acr, 2-methoxy-6-chloro-9-aminoacridine; (OP)$_2$Cu$^+$, 2:1 1,10-phenanthroline–cuprous ion complex; 27-mer, 5′-TGAGTGAGTAAAAAAAATGAGTGCCAA-3′.

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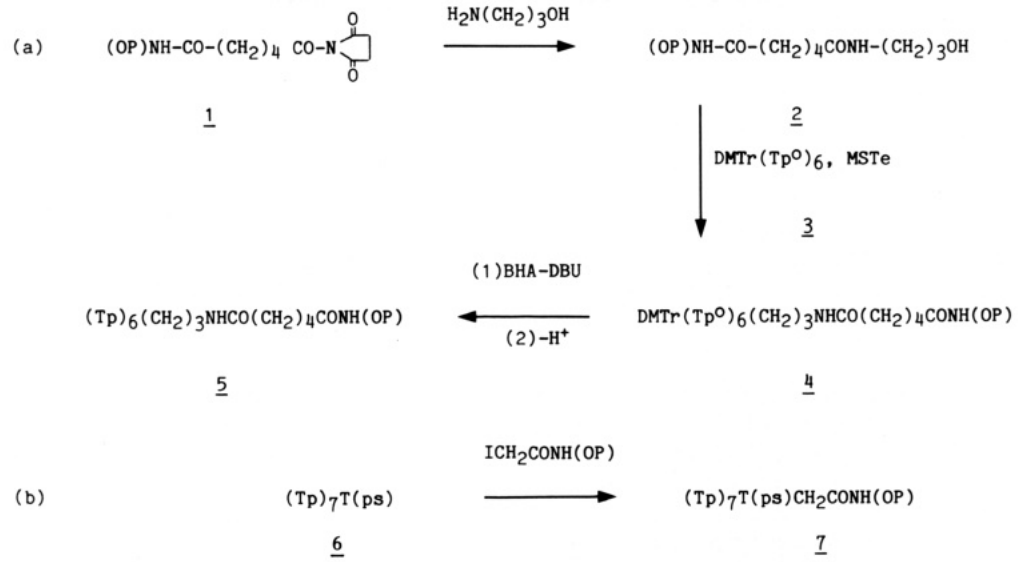
Scheme I: Synthesis of Oligothymidylates Covalently Linked to 1,10-Phenanthroline[a]



[a] BHA, benzohydroxamic acid; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DMTr, dimethoxytrityl; MSTe, mesitylenesulfonyl tetrazolide; OP, 1,10-phenanthroline; p°, chlorophenyl phosphoester; ps, thiophosphate; T, thymidine.

phenanthroline (5 equiv) was stirred at room temperature for 1 h in a solution containing DMSO, H_2O, and 5% $NaHCO_3$ (2:2:1 v/v). T_8-(OP) was then purified by ion-exchange chromatography.

Cleavage Reactions. Cleavage reactions were performed in Eppendorf tubes containing buffered solutions (100 mM NaCl, 50 mM phosphate, pH 7.4) of the polynucleotide or oligonucleotide and the OP derivative. Distilled water and all solutions other than metal salts were treated with chelex. The cleavage reaction was then initiated by the addition of cupric sulfate, followed by mercaptopropionic acid (MPA). The final volume was 5 or 10 μL. This reaction was carried out at 4 °C for 2 h and then quenched by addition of 2,9-dimethyl-OP. The samples were lyophilized and then redissolved in Maxam–Gilbert loading buffer (Maxam & Gilbert, 1980) (80% deionized formamide containing 0.1% xylene cyanol and 0.1% bromophenol blue). They were heated at 90 °C for 1 min, loaded onto 8%, 12%, or 25% polyacrylamide/7 M urea, 29:1 cross-linked gels, and electrophoresed at 45 W for 2 h. The gel was exposed to Kodak or Fuji X-ray film at −70 °C with an intensifying screen or at −20 °C without intensifying screen.

The extent of cleavage was determined by comparing the radioactivity of the intact polymer to that of the degraded polymer by counting the corresponding bands excised from the gel. Microdensitometry of autoradiograms was performed on a Helena densitometer.

Results

The 1,10-phenanthroline–cuprous complex $[(OP)_2Cu^+]$ cleaves DNA in a reaction that requires oxygen and a reducing agent (Sigman & Graham, 1979; Marshall et al., 1981). Sigman et al. have shown that neither single-stranded poly(dA) nor single-stranded poly(dT) was cleaved by $(OP)_2Cu^+$ under conditions where poly(dA)·poly(dT) and alternating poly(dA-T) were completely degraded. These authors also showed that the 2:1 complex and not the 1:1 complex was involved in the cleavage reaction (Marshall et al., 1981). Covalent linkage of 1,10-phenanthroline to an oligonucleotide was expected to bring the cleaving reagent in close proximity to the complementary sequence and therefore to confer upon this molecule a targeted nucleolytic activity (Chen & Sigman, 1986).

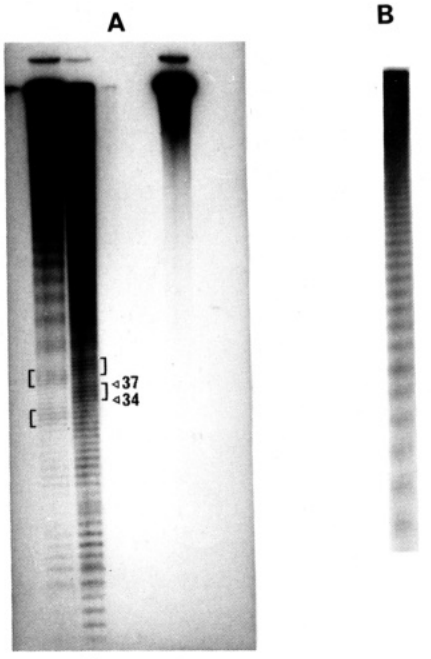


FIGURE 1: (A) Scission of 3′-labeled poly(dA) by T_6-(OP) and T_8-(OP). Poly(dA) (20 μM) was incubated for 2 h at 4 °C with 20 μM T_8-(OP), 10 μM Cu^{2+}, and 2 mM MPA (lane a); 20 μM T_6-(OP), 10 μM Cu^{2+}, and 2 mM MPA (lane b), and 20 μM T_8-(OP) and 10 μM Cu^{2+} (lane c). (B) Scission of 5′-labeled poly(dA) by T_8-(OP) (same conditions as in (A).

Periodic Cleavage of Poly(dA). The cleavage of poly(dA) by T_6-(OP) and T_8-(OP) in the presence of $CuSO_4$ and MPA is presented in Figure 1A. In the presence of 20 μM T_6-(OP) or T_8-(OP) (in nucleotide units), 10 μM cupric ion and 2 mM mercaptopropionic acid cleavage of 3′-labeled poly(dA) (20 μM) led to a family of bands exhibiting a periodic pattern. As shown in Figure 1B the periodicity extended very far. More than 30 peaks could be counted on the densitometric tracing of the gel. These families of bands did not migrate at the same position when the cleavage reaction was carried with either T_6-(OP) or T_8-(OP) (Figure 1, lanes a and b). Analysis of the band distribution in the gels of Figure 1A (see also Figure 2B) indicated that on the average two consecutive bands were

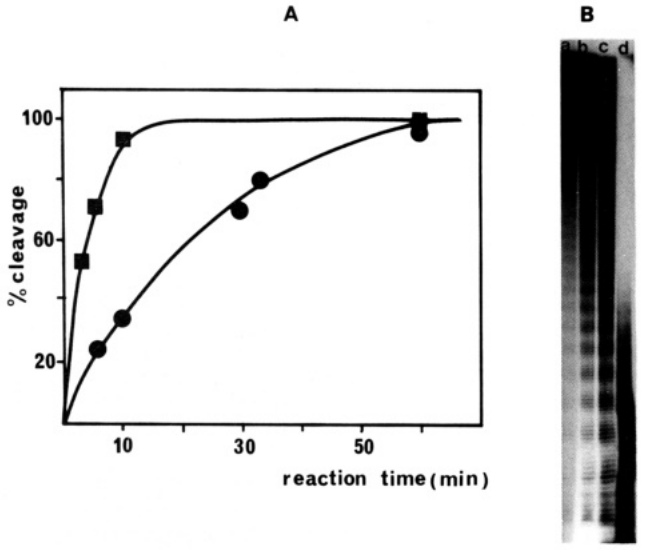


FIGURE 2: Kinetics of cleavage of poly(dA) by T_8-(OP). (A) 3′-Labeled poly(dA) (20 μM) was incubated with 20 μM T_8-(OP), 1 μM $CuSO_4$, and 2 mM MPA at 4 °C in the absence of free OP (●) or in the presence of 2.5 μM OP added to the reaction mixture prior to the addition of $CuSO_4$ and MPA (■). (B) Autoradiogram of high-resolution denaturing gel from which the kinetics of cleavage of poly(dA) incubated with T_8-(OP), OP, $CuSO_4$, and MPA was measured. Lanes a–d correspond to incubation times of 3, 6, 10, and 60 min.

separated by eight and six nucleotides when the cleavage was induced by T_8-(OP) and T_6-(OP), respectively. No cleavage of poly(dA) was observed if MPA was omitted (Figure 1, lane c). The reaction conditions adopted involved the hybridization of T_6-(OP) or T_8-(OP) to poly(dA) prior to the addition of cupric ion and MPA in order to initiate the reaction when required. Cupric ions were added last because of the possibility that they would be chelated by two phenanthrolines belonging to two different oligonucleotides forming a complex structure that would not hybridize efficiently. Despite the fact that the polynucleotide sample used in our experiments was polydisperse, the band ladder was observed when either the 5′- or the 3′-end was labeled (Figure 1). This suggested that (i) the binding of T_6-(OP) and T_8-(OP) to poly(dA) was cooperative, (ii) the nicking sites were localized at well-defined positions with respect to the 3′-end of the oligothymidylates, and (iii) the time required for the cleavage reaction was less than the residence time of the oligonucleotide on the poly(dA) matrix.

Kinetics of Cleavage of Poly(dA) and Stoichiometry of the Coordination Complex in Cleavage Reaction. The time course of cleavage of poly(dA) by T_8-(OP) in the presence of 2 mM MPA at 4 °C is presented in Figure 2. The extent of reaction increased with time and reached a plateau after about 1-h incubation. Under the concentration conditions indicated in the legend of Figure 2, 100% of the poly(dA) could be cleaved. Under the same conditions poly(dT) was not cleaved, indicating that the reaction was specific for the complementary sequence of the oligonucleotide. Similar kinetics were observed when T_6-(OP) was used instead of T_8-(OP) to cleave poly(dA) at 4 °C. When exogenous (OP) was added to the reaction mixture containing T_8-(OP), Cu^{2+}, and MPA (for details, see Figure 2), the rate of the cleavage reaction was enhanced. These results suggested that two phenanthrolines were essential for the generation of the reactive species. The success in targeting the cleavage in the absence of exogenous (OP) tends to support the view that one of the phenanthrolines was contributed by a nonhybridized oligonucleotide–OP. The results presented in Figure 2 also demonstrated that exogenously

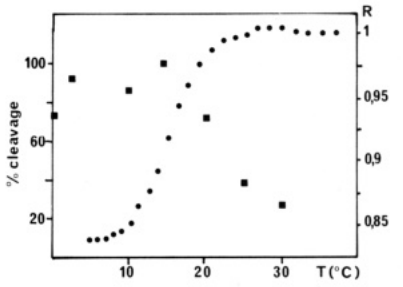


FIGURE 3: Specific cleavage of poly(dA) by T_8-(OP) as a function of temperature (■). The concentrations of poly(dA), T_8-(OP), Cu^{2+}, and MPA were the same as those indicated in Figure 1. Melting profile for the above complex (●); R = absorbance of the mixture at 268 nm divided by the sum of the absorbances of poly(dA) and T_8-(OP).

added OP interacted with oligonucleotide–OP bound to the polynucleotide since the cleavage pattern was similar to that obtained with T_8-(OP) alone. This was also supported by the observation that OP alone at the same concentration was unable to cleave poly(dA) or a poly(dA)–$(dT)_8$ complex.

Temperature Dependence of the Cleavage Reaction. The reaction rate for the cleavage of poly(dA) by T_8-(OP) remained constant between 0 and 10 °C. Above 15 °C the rate dropped rapidely (Figure 3). The decrease in the reaction rate reflected the dissociation of the oligonucleotide from its polynucleotide matrix as measured from the temperature dependence of the absorbance at 260 nm (Figure 3). Dissociation of the poly(dA)–T_8-(OP) complex was observed with a half-transition temperature of 16 °C. The results presented in Figure 3 indicated that efficient cleavage was still observed when the concentration of bound T_8-(OP) was rather low. The rate of cleavage was measured by the decrease in the intensity of the intact poly(dA) band on polyacrylamide gels. A single cleavage reaction on a poly(dA) chain was therefore sufficient to be included in the calculated cleavage efficiency. The rate of cleavage of DNA chains by free $(OP)_2Cu^+$ was previously shown to increase with temperature. Taken together these two effects explain why the temperature at which the cleavage efficiency was reduced to 50% of its maximum value was higher (23 °C) than that of the melting temperature (16 °C) obtained from absorbance measurements. It should also be noted that the active complex involves Cu^{2+} bound to T_8-(OP), which might slightly increase the melting temperature by bringing positive charges close to poly(dA). When the temperature was raised, not only was the rate of cleavage reduced but also the periodic pattern observed at low temperature disappeared.

Cleavage of [5′-^{32}P]27-mer. Figure 4 shows the polyacrylamide gel analysis of the fragments obtained when [5′-^{32}P]27-mer (50 nM) was reacted with 5 μM T_8-(OP). Cupric ions were added after hybridization of 27-mer and oligonucleotide–OP. The most efficient cleavage occurred at A_7, G_8, A_{10}, and A_{11}. The larger number of nicked sites obtained with T_6-(OP) reflected the sliding of this oligonucleotide along the target sequence (data not shown). It should be noted that the fragments obtained with the 5′-labeled 27-mer migrated at the same position as those generated during the Maxam–Gilbert sequencing procedure, indicating that 3′-phosphate termini were generated during the cleavage reaction.

Analysis of Cleavage Pattern Generated by T_8-(OP) Bound to Poly(dA). The pattern of poly(dA) cleavage by T_8-(OP) was analyzed in more detail. It was previously reported that oligothymidylates bind cooperatively along a poly(dA) matrix. Covalent linkage of 1,10-phenanthroline to oligothymidylates did not impair cooperative binding as indicated by the cooperativity of the melting curves (Figure 3) and by the very

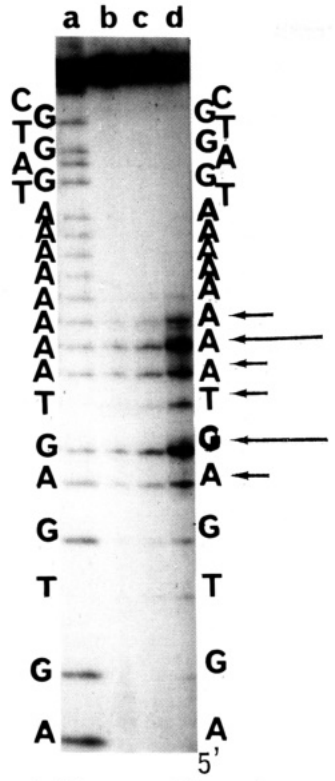


FIGURE 4: Scission of a 27-mer containing an A_8 sequence by T_8-(OP). Cleavage of [5′-^{32}P]27-mer incubated with 5 μM T_8-(OP), 1 μM $CuSO_4$, and 2 mM MPA at 4 °C for 15 (b), 30 (c), and 90 min (d). Maxam–Gilbert (G+A) reaction (lane a).

existence of periodic cleavage. Random binding of T_8-(OP) to a poly(dA) matrix could not give rise to a repeated pattern of cleavage reactions. The observation that the periodicity was obtained when the polynucleotide was either 5′ or 3′ end labeled suggested that it could not result from a phasing of the octathymidylates with respect to one of the polynucleotide ends. The poly(dA) sample used in our experiments was polydisperse, precluding a complete coverage of polynucleotide chains by T_8-(OP). Therefore, the results were analyzed by assuming that cooperatively bound T_8-(OP) could slide along the polynucleotide matrix and that all positions with respect to either end of the polynucleotide were occupied with equal probability. The phenanthroline–copper complex attached at the 3′-end of the octathymidylate can cleave the phosphodiester backbone of its target sequence at different positions as revealed by the analysis of the cleavage pattern of the 27-mer (Figure 4). The environment of OP in cooperatively bound T_8-(OP) along a poly(dA) matrix is obviously different from that of T_8-(OP) bound to the single $(dA)_8$ sequence included in the 27-mer. Therefore, the distribution of cleaved phosphodiester bonds arising from reactions induced by individual T_8-(OP) along the poly(dA) matrix was expected to be different from that observed with the 27-mer. The experimental distribution observed along the poly(dA) matrix was therefore fitted to (i) a distribution of cleavage sites for each T_8-(OP), (ii) a distribution of T_8-(OP) aggregates along the polynucleotide matrix arising from sliding, and (iii) a distribution of polynucleotide length. The fit was calculated for the first "waves" of cleavage of poly(dA) labeled at its 5′-end (Figure 5A). The length of the matrix was written as $8n + k$ with $0 \leq k \leq 7$. For a given n, all values of k were assumed to have equal probabilities. The average length of poly(dA) was long enough (900–1000 nucleotides) so that different n values would give the same distribution of cleavage sites. For a polynucleotide chain containing $8n + k$ nucleotides T_8-(OP)

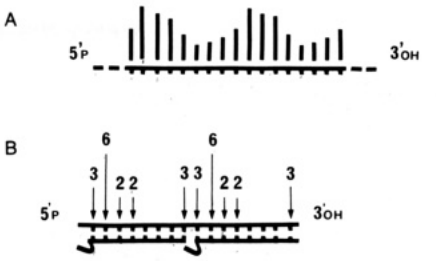


FIGURE 5: (A) Histogram of poly(dA) cleavage produced by T_8-(OP). Bar lengths represent the extent of cleavage resulting from the reaction at the indicated nucleotide. (B) Cleavage sites around the OP of a T_8-(OP) molecule bound to a $(dA)_8$ site on poly(dA), calculated to fit the experimental distribution shown in (A) according to the model described in the text. Numbers represent relative efficiencies of cleavage reactions.

clusters can occupy $k + 1$ positions with respect to the labeled end. Each position was assumed to be occupied with equal probability. According to this model, the experimental distribution of cleavage sites along the poly(dA) matrix was only dependent on the assumed distribution of cleavage sites around the OP of a single T_8-(OP) molecule bound to a $(dA)_8$ site on poly(dA). The best fit was obtained with the distribution shown in Figure 5B. As expected (see above), this distribution is different from that observed when a single T_8-(OP) molecule is bound to a *single* $(dA)_8$ site (e.g., on the 27-mer) (Figure 4).

Cleavage of the phosphodiester backbone occurs both on the 5′-side and on the 3′-side of a nucleoside whose deoxyribose has reacted with the OH$^\bullet$ radicals produced by OP–copper complexes. Therefore, the patterns of poly(dA) cleavage are expected to be shifted with respect to each other depending on whether the 5′-end or the 3′-end is labeled. In a dA_8·dT_8-(OP) duplex the nucleoside which reacts with the highest probability is located at the 7th position from the 3′-end of the $(dA)_8$ sequence, as shown in Figure 5B. Cleavage at this position generates a fragment containing six adenine bases. The calculated cleavage distribution shown in Figure 5B predicts that the fragments produced with the highest frequency (peaks in the distribution of cleavage products) when the 5′-end of poly(dA) is labeled should have lengths of $8p + 1$ nucleotides, where p is an integer. When the 3′-end is labeled, these fragments should be $8p - 2$ in length. It should be noted that the fragments generated when poly(dA) is labeled at its 5′-end have a phosphate at both their 5′-end and their 3′-end. When poly(dA) is labeled by terminal nucleotidyl transferase with [α-^{32}P]ddATP, the fragments have a single phosphate at the 5′-end. The length markers used in our experiments were $p(dA)_8$, $p(dA)_6$, and the products of $p(dA)_8$ digestion according to Maxam and Gilbert (see Figure 6). The lengths of the fragments generated by T_8-(OP) cleavage of poly(dA) were determined when either the 5′-end or the 3′-end was labeled. As shown in Figure 6, the center of gravity of the cleavage bands was displaced by three nucleotide units when the two ladders were compared, in good agreement with the prediction based on the calculated distribution of cleavage sites for T_8-(OP) bound to poly(dA).

DISCUSSION

The results presented in this study demonstrate that oligonucleotides covalently linked to 1,10-phenanthroline induced cleavage reactions in a complementary polynucleotide and a 27-mer containing the complementary sequence in the presence of copper ion and a reducing agent. The cleavage efficiency was higher at low temperature when the complexes were stable. Upon increasing the temperature, the reaction yield decreased

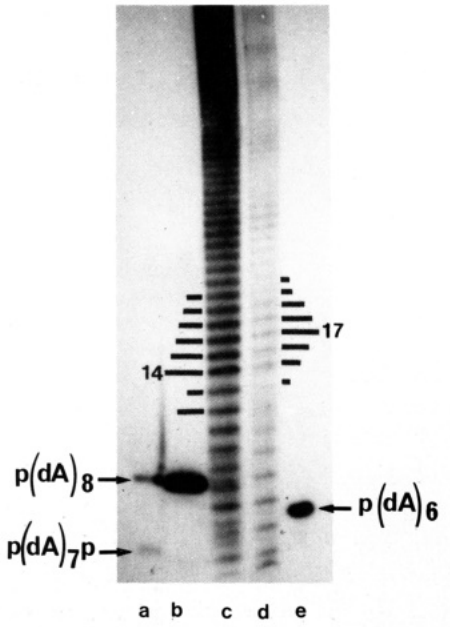


FIGURE 6: Scission of 3′- and 5′-labeled poly(dA) by T_8-(OP). Poly(dA) (20 μM) was incubated for 2 h at 4 °C with 20 μM T_8-(OP), 10 μM Cu^{2+}, and 2 mM MPA. 3′-Labeled poly(dA) (lane c); 5′-labeled poly(dA) (lane d). 5′-Labeled $(dA)_8$ (lane b) and $(dA)_6$ (lane e). Lane a is the Maxam–Gilbert digest of $(dA)_8$. Bar length represents the extent of cleavage resulting from reaction of the indicated nucleotide calculated from the densitometer traces of gel autoradiograms.

as a result of dissociation of the oligonucleotide–polynucleotide complex. But the most striking result remains the series of bands corresponding to a repeat unit obtained when T_6-(OP) or T_8-(OP) reacts with poly(dA) in the presence of Cu^{2+} and MPA. This periodicity reveals indirectly that the binding of OP-substituted oligonucleotides to poly(dA) is cooperative and also that the hydroxyl radicals produced by $(OP)_2Cu^+$ are not freely diffusing along the polymer. The localized nicking sites observed when T_8-(OP) cleaves a 27-mer containing the target A_8 sequence also suggest that the radical species generated by $(OP)_2Cu^+$ are less freely diffusible than those produced by other systems such as EDTA–Fe(II) (Chu & Orgel, 1985; Dreyer & Dervan, 1985).

Although no exogenous OP was required for sequence-specific cleavage of poly(dA) and 27-mer by T_6-(OP) and T_8-(OP) in the presence of Cu^{2+} and MPA, the cleavage reaction was enhanced in a specific manner when exogenous OP was added to the reaction mixture. Previous studies (Marshall et al., 1981) demonstrated that the 2:1 complex $(OP)_2Cu^+$ is the reactive nucleolytic form of the OP–copper chelates, while the 1:1 complex is inert. Our results show that two phenanthrolines are required for the generation of the reactive oxidative species, one of them contributed by a nonhybridized oligonucleotide–OP. The formation of $(OP)_2Cu^+$ complex is certainly less efficient when the second phenanthroline is carried by a negatively charged oligonucleotide due to the repulsive electrostatic interaction between the two OP-carrying molecules. Our results confirm that hybridization may be used to direct a destructive reagent to a specific sequence in a single-stranded DNA. This permits a sequence-dependent cleavage of the target.

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Registry No. **1**, 112863-62-4; **2**, 112863-63-5; **4**, 112895-71-3; **5**, 112863-64-6; **6**, 112895-72-4; **7**, 112863-65-7; 5′-TGAGTGAG-TAAAAAAAATGAGTGCCAA-3′, 112603-07-3; DMTr$(Tp^o)_6$, 65062-64-8; 3-aminopropanol, 156-87-6; poly(dA), 25191-20-2; 5-(iodoacetamido)-1,10-phenanthroline, 111047-29-1.

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