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Sequence-Targeted Photosensitized Reactions in Nucleic Acids by Oligo-α-deoxynucleotides and Oligo-β-deoxynucleotides Covalently Linked to Proflavin†

Danièle Praseuth,‡ Trung Le Doan,‡ Marcel Chassignol,§ Jean-Luc Decout,‖ Noureddine Habhoub,‖ Jean Lhomme,‖ Nguyen T. Thuong,§ and Claude Hélène*,‡

Laboratoire de Biophysique, INSERM U.201, CNRS UA.481, Muséum National d'Histoire Naturelle, 61 Rue Buffon, F-75005 Paris, France, Centre de Biophysique Moléculaire, CNRS, F-45071 Orléans Cedex 02, France, and Laboratoire de Chimie Organique et Macromoléculaire, CNRS UA.351, Université des Sciences et Techniques de Lille I, UER de Chimie, F-59665 Villeneuve d'Ascq Cedex, France

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ABSTRACT: Proflavin was covalently linked to the 3′-end or to the 5′-end of an octadeoxythymidylate. This oligonucleotide was synthesized with either the natural β-anomer of thymidine or its synthetic α-anomer. A polymethylene chain was used to link one of the amino groups of proflavin to a terminal thiophosphate group of the oligonucleotide. A 27-mer oligodeoxynucleotide containing an octadeoxyadenylate sequence was used as a target for the proflavin-substituted octadeoxythymidylates. Upon irradiation with visible light, photo-cross-linking reactions induced the formation of branched species that migrated more slowly than the 27-mer on denaturing polyacrylamide gels. Piperidine treatment of the photo-cross-linked species induced strand breaks in the 27-mer. In addition, proflavin induced photosensitized reactions at guanine residues in the 27-mer sequence which were converted to strand breaks following piperidine treatment. Triple-helix formation by the oligothymidylates with their complementary oligodeoxyadenylate sequence at high salt concentration led to photo-cross-linking and cleavage reactions on both sides of the target sequence. These results show that it is possible to target photosensitized reactions to specific sequences on nucleic acids. This opens new possibilities for site-directed mutagenesis and the development of photoactive anti-messenger oligodeoxynucleotides.

The recent development of anti-messenger oligonucleotides or anti-sense RNAs has opened new possibilities for the specific regulation of gene expression (Green et al., 1986). Highly specific complexes can be formed by short oligonucleotides with their complementary sequence. A 12-mer should find only one target sequence in the *Escherichia coli* genome, assuming a random distribution of base pairs and an equal number of A·T and G·C base pairs. The human genome contains more A·T than G·C base pairs. To specify a site that reappears at random with a frequency less than unity, the minimal oligonucleotide length varies between 15 and 19, depending on whether the oligonucleotide contains only G and C or A and T, respectively (Hélène & Thuong, 1987). Only a small fraction of the genome is transcribed as messenger RNA in a living cell. If the oligonucleotide is targeted to a mRNA, its length can be further decreased without loss of specificity. Assuming that about 0.5% of the genome is expressed as messages in a given cell type, the minimal oligonucleotide

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‡ Muséum National d'Histoire Naturelle.
§ Centre de Biophysique Moléculaire, CNRS.
‖ Université des Sciences et Techniques de Lille I, UER de Chimie. Present address: Laboratoire d'Etudes Dynamiques et Structurales de la Sélectivité, CNRS UA.332, Université de Grenoble I, B.P. 68, F-38402 Saint-Martin d'Hères, France.

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length is reduced from 15–19 to 11–15. Additional constraints may further decrease the minimal length required to obtain specific hybridization of an oligonucleotide to its target sequence in the mRNA population. For instance, in eukaryotes the dinucleotide CpG occurs with a lower frequency than that expected on the basis of a random distribution of base pairs. Therefore, a short oligonucleotide containing one (or more) CpG sequence(s) will have a low probability of finding several complementary sequences in a human genome. However, reduction of the oligonucleotide size decreases the stability of the complex formed with a complementary sequence. We previously showed that the stability could be increased without loss of specificity by covalent attachment of an intercalating agent to the 3′- or 5′-end of the oligonucleotide (Asseline et al., 1983, 1984a,b; Hélène et al., 1985; Lancelot et al., 1985). Such molecules have been used to selectively inhibit mRNA translation (Hélène et al., 1985; Toulmé et al., 1986; Cazenave et al., 1987).

The binding of oligonucleotide–intercalator conjugates to their target sequence is reversible, and thus it is difficult to achieve 100% inhibition. In order to make the process irreversible, chemical reagents can be attached to the oligonucleotide in order to cross-link the oligonucleotide to its complementary sequence or to cleave the phosphodiester bonds in the target nucleic acid (Knorre & Vlassov, 1985; Chu & Orgel, 1985; Dreyer & Dervan, 1985; Boidot-Forget et al., 1986; Chen & Sigman, 1986; Le Doan et al., 1986, 1987a,b). Here we show that site-directed irreversible reactions can be induced in single-stranded nucleic acids by visible light provided a photosensitizer is covalently attached to the oligonucleotide. We have used proflavin as both an intercalating agent and a photosensitizer.

Oligonucleotides can be synthesized with either the natural β-anomers of the nucleoside units or the synthetic α-anomers where the base is inverted with respect to the deoxyribose moiety (Holy, 1973; Sequin, 1974; Morvan et al., 1986; Thuong et al., 1987; Sun et al., 1987). Oligo-α-deoxynucleotides are resistant to nucleases, which makes them very interesting tools for in vivo studies (Thuong et al., 1987; Morvan et al., 1987). They form a double helix with a complementary natural DNA sequence in which the two strands have a parallel orientation (Praseuth et al., 1987, 1988; Sun et al., 1987). We have covalently attached proflavin to an oligo-α-deoxynucleotide, and we show that this molecule can be used to direct photosensitized reactions to a complementary DNA sequence.

Materials and Methods

The different steps involved in the synthesis of the proflavin derivatives used in our experiments are described in Figure 1. Melting points were determined on a Tottoli apparatus and are uncorrected. The ^{1}H NMR spectra were recorded on a Bruker WP-60 (60-MHz) spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to hexamethyldisiloxane as an internal reference. Mass spectra (MS) were recorded on a Riber Mag 10-10 or a Kratos MS-50 radio frequency spectrometer. Infrared (IR) and UV spectra were obtained by using a Perkin-Elmer 237 and a Lambda 5 spectrometer, respectively. Elemental analyses were performed by Service Central de Microanalyses du CNRS (Solaise, France).

3-Acetamido-6-aminoacridine (**2**). To a solution of proflavin (**1**) (1.00 g; 4.78 mmol) in propionic acid (70 mL) cooled at −20 °C was added acetic anhydride (6 mL; 64 mmol) with stirring. After 11 h, the solution was poured into a petroleum ether–diethyl ether mixture (80:20, 250 mL). The precipitate

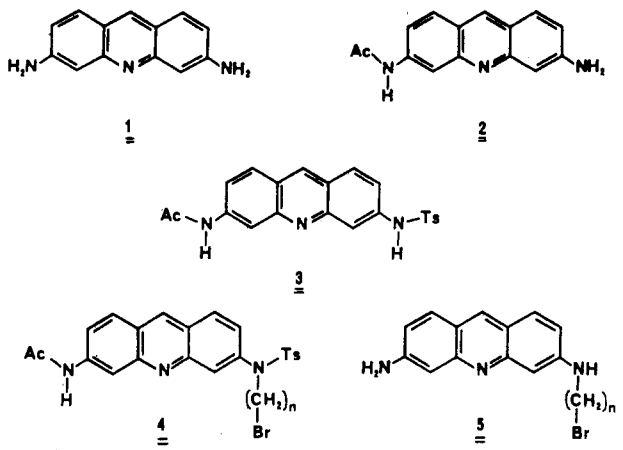

FIGURE 1: Scheme for the synthesis of 3-amino-6-[(ω-bromoalkyl)-amino]acridine (see Materials and Methods).

was filtered, washed with diethyl ether, and dried. This salt was dissolved in water (250 mL) and then precipitated as the free base by addition of concentrated ammonium hydroxide. The precipitate was filtered, washed with water, and dried (1.00 g; 3.98 mmol; 83%): mp 282–285 °C; ^{1}H NMR [60 MHz, dimethyl sulfoxide-d_6 (DMSO-d_6)] δ 10.20 (1 H, s, N*H*COCH$_3$), 8.50 (1 H, s, C$_9$*H*); 8.30 (1 H, d, J = 1.7 Hz, C$_4$*H*), 7.85 (1 H, d, J = 9 Hz, C$_1$*H*), 7.70 (1 H, d, J = 9 Hz, C$_8$*H*), 7.40 (1 H, dd, J = 9 Hz, J = 1.7 Hz, C$_2$*H*), 6.95 (1 H, dd, J = 9 Hz, J = 1.8 Hz, C$_7$*H*), 6.85 (1 H, d, J = 1.8 Hz, C$_5$*H*), 6.00 (2 H, s, N*H*$_2$), 2.15 (3 H, s, COC*H*$_3$); MS [electron impact (EI)] m/z 251 (M$^+$, 1.5), 236 (M$^+$ − CH$_3$, 1.5).

3-Acetamido-6-(p-toluenesulfonamido)acridine (**3**). To a solution of **2** (3.00 g; 11.9 mmol) in pyridine (100 mL) cooled to 4 °C was added dropwise a solution of *p*-toluenesulfonyl chloride (6.81 g; 35.7 mmol) in pyridine (15 mL) with mechanical stirring. The precipitate formed was dissolved by addition of triethylamine (4 mL). After 2 h, a second part of *p*-toluenesulfonyl chloride (14.7 g; 77 mmol) in pyridine (10 mL) was added. The mixture was kept at 4 °C with stirring for 18 h and then concentrated to dryness under reduced pressure. To the residue dissolved in absolute ethanol (10 mL) was added ethyl acetate (200 mL). From the solution kept overnight at 0 °C was removed by filtration triethylamine hydrochloride which crystallized. The filtrate was evaporated to dryness under reduced pressure, and diethyl ether was added. The insoluble residue was filtered and washed. It contained a mixture of **3** and a derivative arising from N,N-ditosylation, 3-acetamido-6-[*N*,*N*-bis(*p*-toluenesulfonyl)-amino]acridine. This latter compound was converted into **3** by a basic treatment of the crude mixture. The solid was dissolved in a water–dimethylformamide (DMF) mixture (6:1), and potassium carbonate was added (2.5 g, 18 mmol). The solution was heated at 100 °C for 14 h and then evaporated to dryness under reduced pressure. The residue was stirred with acetone, and the insoluble mineral material was filtered off. From the filtrate concentrated under reduced pressure was precipitated **3** by addition of diethyl ether. It was filtered, washed, and dried (3.62 g; 72%): mp 180 °C dec; ^{1}H NMR (60 MHz, DMSO-d_6) δ 10.35 (1 H, br s, N*H*COCH$_3$), 8.75 (1 H, s, C$_9$*H*), 8.45 (1 H, br s, C$_4$*H*), 8.05–7.20 (9 H, m, C$_1$*H*, C$_2$*H*, C$_5$*H*, C$_7$*H*, C$_8$*H*, Ts*H*), 2.25 (3 H, s, TsC*H*$_3$), 2.10 (3 H, s, COC*H*$_3$); MS (EI) m/z 405 (M$^+$, 5), 250 (M$^+$ − Ts, 33). Anal. Calcd for C$_{22}$H$_{19}$N$_3$O$_3$S·H$_2$O: C, 62.40; H, 5.00; N, 9.92; S, 7.57. Found: C, 62.56; H, 4.93; N, 9.94; S, 7.34.

3-Acetamido-6-[N-(p-toluenesulfonyl)-N-(ω-bromoalkyl)amino]acridine [**4a** (n = 3) *and* **4b** (n = 5)]. To a

solution of **3** (1.00 g; 2.47 mmol) in anhydrous DMF (45 mL) were successively added potassium carbonate (2 g; 14.5 mmol) and α,ω-dibromoalkane (25 mmol). The mixture was stirred at room temperature for 24 h and then filtered to remove the insoluble mineral salts. The filtrate was concentrated to dryness under reduced pressure. The residue was chromatographed on silica gel (elution with dichloromethane–ethyl acetate mixtures). Crystallization from ethyl acetate afforded the pure compounds **4a** and **4b**.

(*A*) *Compound* **4a** (*n* = *3*). Yield: 71% (0.92 g; 1.75 mmol). mp 193–194 °C. ^{1}H NMR (60 MHz, DMSO-d_6) δ 10.40 (1 H, s, N*H*), 8.95 (1 H, s, C_9H), 8.50 (1 H, br s, C_4H), 8.50–7.30 (9 H, m, Ts*H*, C_1H, C_2H, C_5H, C_7H, C_8H), 4.25–3.00 (4 H, m, N–CH_2, CH_2–Br), 2.35 (3 H, s, TsCH_3), 2.20 (3 H, s, COCH_3), 2.00 (2 H, m, CH_2–CH_2–CH_2). Anal. Calcd for $C_{25}H_{24}BrN_3O_3S$: C, 57.04; H, 4.59; N, 7.98; Br, 15.18. Found: C, 56.99; H, 4.64; N, 8.06; Br, 15.17.

(*B*) *Compound* **4b** (*n* = *5*). Yield: 58% (0.79 g; 1.42 mmol). mp 166–167 °C. ^{1}H NMR (60 MHz, DMSO-d_6) δ 10.40 (1 H, s, N*H*), 8.95 (1 H, s, C_9H), 8.50 (1 H, br s, C_4H), 8.10 (2 H, d, J = 9.0 Hz, C_1H, C_8H), 7.70 (1 H, br s, C_5H), 7.60 (1 H, d, C_2H), 7.40–7.25 (5 H, m, C_7H, Ts*H*), 2.45 (4 H, m, N–CH_2, CH_2–Br), 2.35 (3 H, s, TsCH_3), 2.15 (3 H, s, COCH_3), 1.40 [6 H, m, CH_2–(CH_2)$_3$–CH_2]. Anal. Calcd for $C_{27}H_{28}BrN_3O_3S$: C, 58.48; H, 5.09; N, 7.58; Br, 14.41. Found: C, 58.23; H, 5.07; N, 7.38; Br, 13.89.

3-Amino-6-[(*ω-bromoalkyl*)*amino*]*acridine* (**5a** *and* **5b**). Compound **4** (1.00 mmol) was dissolved in concentrated sulfuric acid (36 N, 2 mL), and then the solution was heated at 80 °C for 2 h. The solution cooled at room temperature was poured dropwise into a diethyl ether–acetone mixture (70:30; 100 mL), and the pure disulfate derivative **5** that precipitated was obtained after filtration and further washed with diethyl ether.

(*A*) *Compound* **5a** (*n* = *3*). Yield: 93%. mp 195–196 °C. ^{1}H NMR (60 MHz, DMSO-d_6) δ 13.50 (1 H, s, N*H*-CH_2), 8.75 (1 H, s, C_9H), 7.80 (2 H, d, J = 9 Hz, C_1H, C_8H), 6.90 (2 H, d, J = 9 Hz, C_2H, C_7H), 6.90 (s with HOD, NH_3^+), 6.65 (1 H, s, C_4H or C_5H), 6.55 (1 H, s, C_5H or C_4H), 3.65 (2 H, t, J = 7 Hz, CH_2-Br or N-CH_2), 3.30 (2 H, t, J = 7 Hz, N-CH_2 or CH_2-Br), 2.10 (2 H, m, –CH_2–CH_2–CH_2). MS [fast atom bombardment (FAB), thioglycerol, acetic acid 1%, positive ions] m/z 332 [M^+(^{81}Br) + 1], 330 [M^+(^{79}Br) + 1], 250 (M^+ – Br), 236 (M^+ – Br – CH_2), 222 (M^+ – Br – 2CH_2). Anal. Calcd for $C_{16}H_{16}BrN_3{\cdot}2H_2SO_4{\cdot}2H_2O$: C, 34.17; H, 4.30; N, 7.47; Br, 14.21. Found: C, 34.15; H, 4.39; N, 7.32; Br, 14.50. UV (ethanol): λ_{max} (ϵ) 464 (57 000), 262 nm (57 000).

(*B*) *Compound* **5b** (*n* = *5*). Yield: 95%. mp 212–213 °C. ^{1}H NMR (60 MHz, DMSO-d_6) δ 13.50 (1 H, s, N*H*–CH_2), 8.70 (1 H, s, C_9H), 7.80 (2 H, d, J = 9 Hz, C_1H, C_8H), 6.95 (2 H, d, J = 9 Hz, C_2H, C_7H), 6.70 and 6.60 (2 H, 2 br s C_4H, C_5H), 3.50 (2 H, t, CH_2–Br or N–CH_2), 3.20 (2 H, t, N–CH_2 or CH_2–Br), 1.55 [6 H, m, CH_2–(CH_2)$_3$–CH_2]. MS (FAB, thioglycerol, positive ions) m/z 360 [M^+(^{81}Br) + 1] 358 [M^+(^{79}Br) + 1], 278 (M^+ – Br), 250 (M^+ – Br – 2CH_2), 236 (M^+ – Br – 3CH_2), 222 (M^+ – Br – 4CH_2), 210. Anal. Calcd for $C_{18}H_{20}BrN_3{\cdot}2H_2SO_4{\cdot}3H_2O$: C, 35.53; H, 4.97; N, 6.91. Found: C, 35.80; H, 4.65; N, 6.57. UV (ethanol): λ_{max} (ϵ) 464 (59 000), 262 nm (59 000).

Oligonucleotide Synthesis. Covalent linkage of the proflavin derivatives **5a** and **5b** to the oligonucleotides was achieved via a terminal thiophosphate group. Oligothymidylates were synthesized with either the natural β-anomers or the synthetic α-anomers of the nucleoside. α-D-Thymidine was obtained from Sigma. The phosphotriester method in solution previously described for β-anomers was adapted to α-anomers (Thuong et al., 1981; Praseuth et al., 1987, 1988).

A thiophosphate group was introduced either at the 5′-end or at the 3′-end of octathymidylates using bis(cyanoethyl) thiophosphate as an intermediate. Introduction of this group on the 5′-side was achieved by reacting the 5′OH group of the protected oligonucleotide with bis(cyanoethyl) *N,N*-diisopropylamidophosphite activated by tetrazole, followed by sulfuration of the intermediate phosphite using sulfur in pyridine. To introduce the thiophosphate group on the 3′-side, a mononucleotide bearing the bis(cyanoethyl) thiophosphate group at the 3′-position and a dimethoxytrityl group at the 5′-position was first synthesized. After detritylation, this substituted monomer was reacted with a protected heptathymidylate 3′-aryl phosphodiester. Deprotection of the phosphate and thiophosphate groups was achieved by treating the oligonucleotides with a mixture of benzohydroxamic acid and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in pyridine and then with sodium hydroxide as previously described (Thuong & Chassignol, 1987). The terminal thiophosphate (1 equiv) was reacted with compound **5a** or **5b** (5 equiv) at room temperature for 20 h in a solution containing DMSO, H_2O, and 5% $NaHCO_3$ (2:2:1 v/v). The same procedure was applied to α- and β-oligothymidylates. Purification was achieved by high-performance liquid chromatography on polyanionic HR 5/5 columns (Pharmacia). Two octathymidylate derivatives synthesized with the natural β-anomers were obtained: one was substituted by compound **5b** at its 3′-end and the other by compound **5a** at its 5′-end. They will be referred to as T_8-Pf and Pf-T_8, respectively. An octathymidylate synthesized with the α-anomer of thymidine was covalently linked to compound **5a** via its 5′-end. It will be abbreviated as Pf-α-T_8.

A 27-mer oligodeoxynucleotide was used as a target for proflavin-substituted oligothymidylates. Synthesis was carried out on either an Applied Biosystems or a Pharmacia automatic synthesizer. The oligonucleotide was purified by gel electrophoresis. Its sequence is (5′)d(TGAGTGAGTAAAAAAA-ATGAGTGCCAA)(3′). It contains an octadeoxyadenylate sequence which constitutes a target for the proflavin-substituted octathymidylates. The 27-mer fragment was 5′-end-labeled by using polynucleotide kinase and [γ-^{32}P]ATP (Amersham).

Results

Photo-Cross-Linking of Proflavin-Substituted Octathymidylates to Their Target Sequence. The proflavin-substituted octadeoxythymidylates were mixed with the 5′-end ^{32}P-labeled 27-mer oligodeoxynucleotide at 20 °C. The solutions were then cooled down to 0 °C, and irradiation was carried out at this temperature. A Pyrex glass filter was used to remove light of wavelengths shorter than 320 nm. The irradiated samples were then loaded at neutral pH on a denaturing 20% polyacrylamide gel. After electrophoresis and autoradiography, new bands were revealed that migrated more slowly than the nonirradiated 27-mer (Figure 2). The relative intensities of these bands were dependent upon the ionic concentration, especially in the case of Pf-T_8 (Figure 2). No cleavage of the 27-mer sequence was observed under neutral conditions. The observation of slowly migrating bands indicated that the oligothymidylate was photo-cross-linked to the 27-mer, thereby forming branched species which were expected to be retarded in the gel. Photosensitized damages that could have been produced on the bases of 27-mer (without photo-cross-linking) were not likely to be revealed on the gel since base damages were not expected to alter markedly the elec-

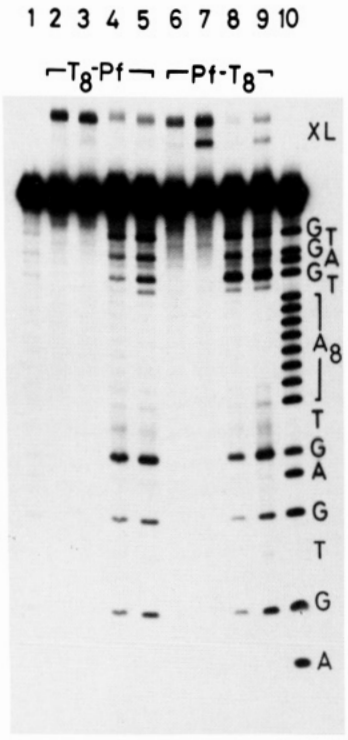

FIGURE 2: Photo-cross-linking and cleavage of the 5′-labeled 27-mer DNA fragment (10 nM) by proflavin-substituted β-octathymidylates (5 μM). In T_8-Pf the proflavin ring is attached to the 3′-end of the octathymidylate. In Pf-T_8 it is attached to the 5′-end. Irradiation was carried out for 10 min at wavelengths longer than 320 nm in a 10 mM sodium phosphate buffer (pH 7) containing either 0.1 or 1 M NaCl. Lane 1 is the unirradiated control treated by 1 M piperidine for 20 min at 90 °C. Lanes 2 and 3 and lanes 6 and 7 correspond to the irradiated samples loaded on a denaturing polyacrylamide gel *without* piperidine treatment. Lanes 4 and 5 and lanes 8 and 9 correspond to the irradiated samples treated by 1 M piperidine for 20 min at 90 °C. NaCl concentrations were as follows: lanes 2, 4, 6, and 8, 0.1 M NaCl; lanes 3, 5, 7, and 9, 1 M NaCl. Lane 10 is the G + A sequence of the 27-mer. Part of the 27-mer sequence is shown at the right. XL indicates photo-cross-linked species.

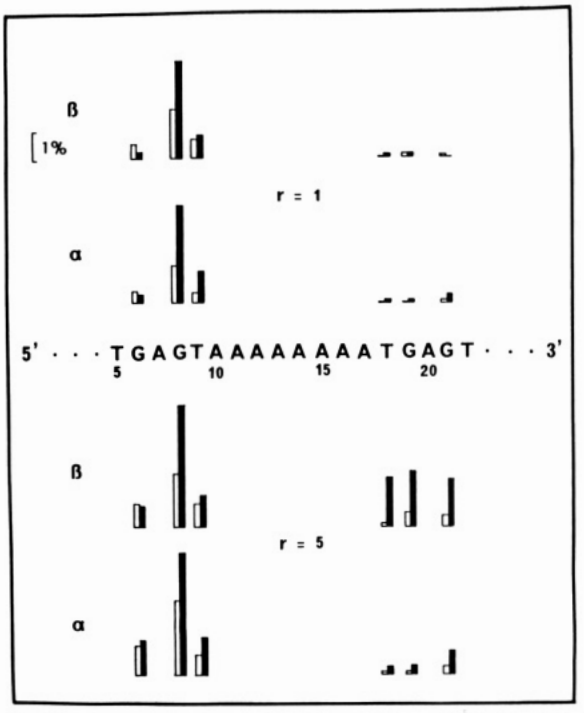

FIGURE 3: Cleavage reactions induced in the 27-mer (1 μM) by β-T_8-Pf or Pf-α-T_8 at 1 (r = 1) and 5 μM (r = 5). The reaction was carried out in 10 mM sodium phosphate buffer, pH 7, containing 0.1 M NaCl (open bars) or 1.0 M NaCl (filled bars). After irradiation at 0 °C the reacted samples were treated by piperidine (1 M) at 90 °C for 20 min and electrophoresed on a denaturing polyacrylamide gel. Bands corresponding to bases adjacent to the A_8 target sequence were cut out of the gel, and radioactivity was measured by scintillation counting. The radioactivity was first corrected for the background obtained with the corresponding blank experiment (27-mer irradiated in the absence of the oligonucleotide) and then divided by the total radioactivity of the lane. The bar scale (left) corresponds to 1% of cleaved products (as compared to the original 27-mer concentration).

trophoretic mobility of the 27-mer.

Cleavage of Photo-Cross-Linked Species under Alkaline Conditions. Previous studies showed that the photo-cross-linked species obtained upon irradiation of *p*-azidophenacyl-substituted oligothymidylates could be cleaved under alkaline conditions (Praseuth et al., 1987, 1988). Even though the photochemical reactions induced by proflavin excitation are different from those obtained with *p*-azidophenacyl, we attempted to use the same strategy to identify the photo-cross-linking sites. The irradiated samples were treated with 1 M piperidine at 90 °C for 20 min and then analyzed on denaturing 20% polyacrylamide gels. As shown on Figure 2, the intensity of the bands originating from photo-cross-linked species decreased, and new bands migrating more rapidly than the original 27-mer were observed. At low ionic concentration (0.1 M NaCl) the most intense band was located at G-8 on the 5′-side of the A_8 sequence of the 27-mer when the oligonucleotide substituted with proflavin at its 3′-end (T_8-Pf) was used as a photosensitizer. When the 5′-substituted oligothymidylate (Pf-T_8) was used as a photosensitizer, the main cleavage reaction occurred at G-19 on the 3′-side of the A_8 sequence. Upon an increase in NaCl concentration from 0.1 to 1 M, cleavage reactions were amplified on opposite sides of the A_8 sequence with both proflavin-substituted oligothymidylates (Figure 2). With T_8-Pf cleavage occurred at T-18, G-19, G-21, and G-23. With Pf-T_8 the main additional cleavage site was at G-8, followed by T-9 and G-6. The difference in cleavage pattern observed at two ionic concentrations suggested the formation of a triple helix which is favored at high salt.

The results presented in Figure 2 were obtained with a large excess of proflavin-substituted octathymidylates (5 μM as compared to 10 nM in 27-mer fragment). An experiment was also carried out at equimolar concentrations (1 μM) of each species. The bands corresponding to cleaved products after piperidine treatment were cut out of the gels and counted for their radioactivity. The results obtained with T_8-Pf are presented in Figure 3. At both 0.1 and 1 M NaCl, cleavage took place exclusively on the 5′-side of the target A_8 sequence. When the concentration of T_8-Pf was increased to 5 μM while the 27-mer concentration was kept at 1 μM, additional cleavage sites were observed on the 3′-side, especially at 1 M NaCl concentrations. The observation of additional cleavage sites on the 3′-side at a 5:1 ratio as compared to a 1:1 ratio is in agreement with the formation of a triple helix where the two octathymidylates have antiparallel orientation with respect to each other (see Discussion).

Analysis of Photo-Cross-Linking and Photooxidation Reactions. The bands corresponding to cross-linked products before piperidine treatment and to cleaved fragments after piperidine treatment were cut out of the gels and the radioactivity was counted in order to estimate the extent of the different photochemical reactions that had taken place in the irradiated samples. The amount of cleaved products was about 3 times as high as that of cross-linked species. For example, when Pf-T_8 (5 μM) was irradiated in the presence of 10 nM 5′-labeled 27-mer at 0.1 M NaCl, the cross-linked products

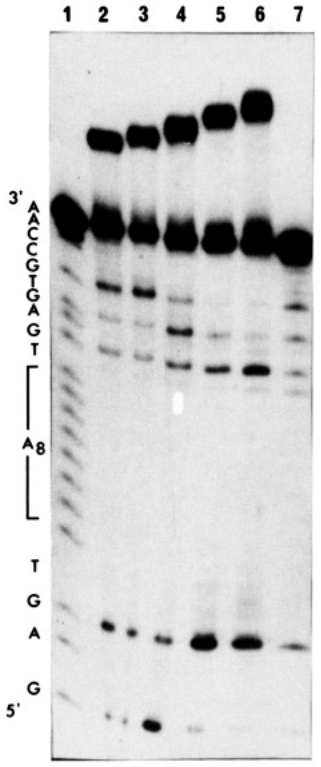

FIGURE 4: Autoradiography of a polyacrylamide gel after electrophoresis of photo-cross-linked species (T_8-Pf + 5'-labeled 27-mer, 0.1 M NaCl) isolated from a gel run under neutral conditions (see Figure 2) and treated by 1 M piperidine at 90 °C for 20 min. Lane 1 is the G + A sequence of the 27-mer. Lanes 2–6 correspond to different fractions cut out of the gel run under neutral conditions. In each lane, the upper band represents the cross-linked fragment which has not been cleaved by piperidine treatment. The bands that migrate as the original 27-mer (or a little bit slower than the 27-mer) result from release of the cross-linked octathymidylate. The bands that appear below the 27-mer fragment reveal cleavage reactions that occur in the 27-mer at the photo-cross-linked sites (mainly guanines). Lane 7 corresponds to the band that migrated at the same position as the intact 27-mer in the gel run under neutral conditions and that was treated by piperidine after excision from the gel. Clevage reactions were induced at all guanines and (weakly) at T-18.

represented 1.9% of the total radioactivity whereas cleaved products (after piperidine) amounted to 6.8%. At 1 M NaCl the corresponding values were 4.4% and 12.2%, respectively. This result suggested that an important fraction of cleavage reactions occurred at damages other than cross-links. Proflavin is known to generate singlet oxygen upon visible light irradiation (Piette et al., 1977) and to induce radical reactions in DNA (Gräslund et al., 1975; Calberg-Bacq et al., 1977). Therefore, part of the cleavage reactions observed after piperidine treatment could occur at photodamaged bases rather than at photo-cross-linked sites. A 27-mer with photooxidized bases but no cross-link would be expected to migrate as the intact 27-mer under neutral conditions.

In order to examine the respective roles of cross-linking and oxidation reactions, the bands corresponding to photo-cross-linked species of T_8-Pf and to the 27-mer were cut out of the gels run under neutral conditions and each of them was treated independently by 1 M piperidine at 90 °C for 20 min. Since the band corresponding to photo-cross-linked species was rather broad, it was separated in several subfractions as shown on Figure 4. Each band corresponding to photo-cross-linked species generated cleaved products in addition to products migrating as the intact 27-mer or slightly slower than the 27-mer. The conversion of the photo-cross-linked species was not complete even after 30 min in the presence of 1 M piperidine at 90 °C, an experiment suggesting that not all cross-linked sites were piperidine-sensitive.

All bands cut out of the gel under neutral conditions generated fragments corresponding to cleavage at guanines located on both sides of the A_8 sequence at high salt concentration. The bands that were more retarded on the gel gave rise to products cleaved at the guanine residues closest to the A_8 sequence (Figure 4, lane 6). Those that were less retarded gave cleavage reactions at guanines more distant from the 3'-side of the A_8 sequence (Figure 4, lanes 2 and 3).

The band migrating as the intact 27-mer on neutral gels gave rise to cleavage reactions at guanines (Figure 4, lane 7). The gels run without alkaline treatment of the irradiated samples revealed that only a few percent of the 27-mer was converted to cross-linked material under our irradiation conditions (Figure 2). The radioactivity present in the "27-mer" band that was extracted from the gel run without piperidine treatment was therefore much higher (20–50 times) than that present in the cross-linked species. To avoid overexposure of the gels, approximately equivalent amounts of radioactivity were loaded on the gel after piperidine treatment of extracted species (Figure 4). Therefore, it should be kept in mind that the sum of the radioactivities of all cross-linked species (lanes 2–6) represented only a few percent of that of the 27-mer band (lane 7) in the original irradiated sample. Consequently, the cleavage at guanines observed upon piperidine treatment of the "27-mer" band represents the most important reaction. Two hypotheses could explain this observation: (i) some of the cross-links were labile at neutral pH and were converted into species that migrated as the intact 27-mer under neutral conditions; (ii) proflavin induced photosensitized reactions in the 27-mer in addition to cross-link formation, thereby leading to a 27-mer containing damaged bases but still migrating at the same location as the intact 27-mer. Even though we have evidence that some of the cross-links are heat-unstable (results not shown), the second hypothesis seems more plausible since it is known that proflavin generates singlet oxygen and can induce formation of peroxyl radicals under visible light irradiation. Guanines are the most sensitive bases to oxidation reactions, and piperidine treatment is expected to induce cleavage reactions at the sites of photooxidized guanines.

Photo-Cross-Linking and Cleavage Reactions Induced by Pf-α-T_8. In order to demonstrate that the octathymidylate synthesized with the α-anomer of thymidine hybridized to its complementary β-sequence, mixtures of the 27-mer (10 nM) and proflavin-substituted α-T_8 were irradiated at 0 °C. Photo-cross-linked species were observed on denaturing gels at pH 7; they migrated similarly to those observed with proflavin-substituted β-T_8 (results not shown). Upon alkaline treatment cleavage was induced at guanine locations as previously observed with β-T_8-Pf (Figure 5). At very low concentration of Pf-α-T_8 (50 nM) and low ionic concentration (0.1 M NaCl) some cleaved products were observed at all guanines (Figure 5, lane 2). They were also observed in the absence of irradiation or if irradiation was carried out in the absence of the proflavin-containing oligothymidylate. They originated from cleavage reactions induced by piperidine treatment of the 27-mer fragment (see also Figure 2). Upon an increase in Pf-α-T_8 and NaCl concentration, cleavage reactions were strongly enhanced at bases located on the 5'-side of the A_8 sequence.

Quantitative analysis of photo-cross-linking and cleavage reactions was carried out at higher concentration of the target 27-mer sequence (1 μM as compared to 10 nM in Figure 5). Bands corresponding to cleaved products were cut out of the

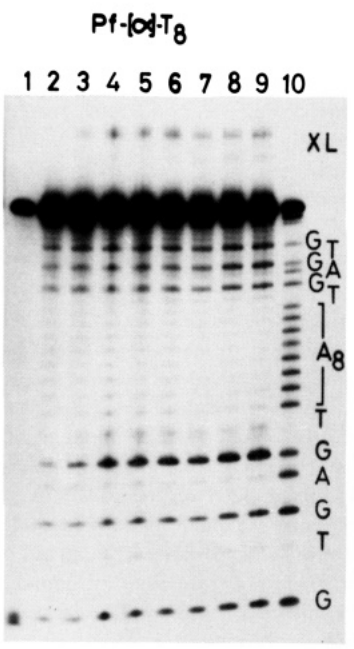

FIGURE 5: Photo-cross-linking and cleavage of the 27-mer DNA fragment (10 nM) by Pf-α-T_8 in a 10 mM sodium phosphate buffer, pH 7. Irradiation was carried out for 15 min at 0 °C (see Materials and Methods). The samples were then treated by 1 M piperidine at 90 °C for 30 min. Lane 1 is the nonirradiated control. Lane 10 is the G + A sequence of the 27-mer. Lanes 2, 3, and 4 correspond to increasing concentrations of Pf-α-T_8, 0.05, 0.5, and 5 μM, respectively, in the presence of 0.1 M NaCl. Lanes 5–9 correspond to increasing NaCl concentrations at 5 μM Pf-α-T_8: lane 5, 0.025 M NaCl; lane 6, 0.1 M NaCl; lane 7, 0.25 M NaCl; lane 8, 0.5 M NaCl; lane 9, 0.75 M NaCl. XL indicates photo-cross-linked species that remain after piperidine treatment of the irradiated samples.

gel and counted for radioactivity. The results are presented in Figure 3 at two different concentrations (1 and 5 μM) of Pf-α-T_8. At low salt concentration (0.1 M NaCl) the cleavage pattern is quite similar to that obtained with β-T_8-Pf. Proflavin is attached to the 3′-end of the oligo-β-deoxynucleotide but to the 5′-end of the oligo-α-deoxynucleotide. These results clearly demonstrate that the α-T_8/β-$(dA)_8$ double helix involves parallel strands in contrast to a double helix built with two oligo-β-deoxynucleotides where the two strands adopt an antiparallel orientation. This result is in agreement with a previous demonstration of the parallel orientation of the two strands in an α–β double helix using *p*-azidophenacyl as a photo-cross-linking reagent (Praseuth et al., 1987, 1988). When salt concentration was increased up to 1 M and the concentration of Pf-α-T_8 up to 5 μM, the cleavage pattern was not markedly affected in contrast to what was observed for β-T_8-Pf (see Figure 3). Triple-helix formation by the oligo-α-deoxynucleotide is weak if it exists at all. We previously showed that α-T_8 substituted by an azidophenacyl group did not form a triple helix even at high oligonucleotide and NaCl concentrations (Praseuth et al., 1987, 1988). However, triple-helix formation was recently observed with α-T_8 covalently linked to an azidoproflavin derivative (Le Doan et al., 1987b).

Discussion

Oligodeoxythymidylates covalently linked to a proflavin derivative form complexes with a complementary oligodeoxyadenylate sequence. Double and triple helices can form, depending on salt concentration. This arises because adenine can form a pair of hydrogen bonds with two different thymines (Riley et al., 1966). The T-A-T triad involves both Watson–Crick and Hoogsteen base pairings. Formation of a triple helix is favored at high salt concentration because electrostatic repulsion between negatively charged molecules decreases when salt concentration increases. Spectroscopic studies (to be published elsewhere) revealed that proflavin-substituted oligothymidylates could form both double and triple helices with complementary oligodeoxyadenylates.

Upon light excitation proflavin induced cross-linking reactions with a single-stranded DNA containing the complementary sequence of the oligonucleotide to which proflavin was tethered. No cleavage was observed at neutral pH. Upon piperidine treatment the photo-cross-linked species were converted to cleaved products. By extraction of the different photo-cross-linked bands from the gel run at neutral pH, it was shown that cleavage reactions occurred not only at cross-linked sites but also at modified guanines resulting from proflavin-photosensitized reactions in the 27-mer. Not all cross-linked sites generated cleaved products since part of the cross-linked materials remained unchanged after treatment by 1 M piperidine for 20 min at 90 °C and part of it was converted to the original 27-mer DNA fragment or to modified products migrating as the 27-mer. The cleaved products migrated at the same position as the DNA fragments generated using the Maxam–Gilbert sequencing procedure (Maxam & Gilbert, 1980). Therefore, they have a phosphate group at their 3′-end. We have no information on the chemical nature of the photo-cross-links involving guanine (or thymine) and proflavin.

At low ionic concentration (0.1 M NaCl) the majority of the cleavage reactions occurred on the 3′-side of the A_8 sequence with Pf-T_8 and on the 5′-side with T_8-Pf. These results are in agreement with the location of proflavin in a duplex involving antiparallel orientation of the two strands. At high salt concentration (1 M NaCl) cleavage on the opposite side of the A_8 sequence was enhanced in agreement with a higher efficiency of triple-helix formation at high salt concentration. The location of the cleavage sites implies that the two T_8 strands in the triple helix have an antiparallel orientation.

When the oligothymidylate was synthesized with the α-anomer of thymidine, a reverse orientation was observed with respect to the A_8 sequence. Proflavin attached to the *5′-end* of α-T_8 was photo-cross-linked to and led to cleavage reactions at guanine and thymine bases on the *5′-side* of the A_8 sequence. This result is in agrement with previous data using α-T_8 covalently linked to a *p*-azidophenacyl group. The location of photo-cross-linking and cleavage reactions unambiguously demonstrated that the α-T_8/β-$(dA)_8$ duplex involved a parallel orientation of the two strands (Praseuth et al., 1987, 1988).

Proflavin has been previously used to photosensitize reactions in DNA (Piette et al., 1981). The damages block DNA replication, and DNA polymerase stops one base before every guanine in the template sequence (Piette & Moore, 1982). This inhibition was assumed to be due to photooxidation products of guanines which were not recognized as normal bases by the polymerase. In light of the results reported in the present study it seems necessary to contemplate the possibility that DNA polymerase was also blocked by guanine–proflavin photoadducts in addition to oxidized guanines.

The most reactive guanines in the T_8–27-mer complexes are those that are immediately adjacent to the target sequence of the proflavin-substituted T_8. However, photo-cross-linking and cleavage reactions were also observed at guanines located further away from the target sequence. These reactions were not due to unspecific reactions induced by proflavin-substituted oligothymidylates not bound to their target sequence since (i) they were not observed when the complexes were dissociated at high temperature (results not shown) and (ii) they had

different efficiencies depending on whether proflavin was attached to the 3′- or the 5′-end of β-T_8 or to the 5′-end of α-T_8. Several factors may contribute to the reactivity of the guanine bases. They are located in a single-stranded region next to the duplex (or triplex) structure involving A_8 and T_8. The flexibility of the phosphodiester backbone might bring these Gs in contact with the photoexcited proflavin. If the cross-linking reaction involves long-lived radical species generated by excitation of proflavin, bending of the single-stranded chain could allow nonadjacent guanines to reach the proflavin radical before it is deactivated. Photoexcited proflavin is also known to generate singlet oxygen (Piette et al., 1977). Unbound oligothymidylates probably do not generate a high enough concentration of singlet oxygen to induce photooxidation reactions at guanines, even at the highest concentration of oligothymidylates used in our experiments (5 μM). On the contrary, oligothymidylates bound to their target A_8 sequence may generate a high local concentration of singlet oxygen in close proximity to the guanines adjacent to the A_8 sequence. As a matter of fact, the reactivity of guanines decreases according to their distance to the A_8 sequence. Studies with longer DNA fragments containing the A_8 sequence are under way to further characterize these reactions.

It should be noted that the two thymines that flank the A_8 sequence have quite different reactivities. Pf-T_8 gives more efficient photo-cross-linking and cleavage reactions at T-18 than T_8-Pf at T-9. This difference in reactivity reveals the important role played by the interactions that proflavin engages in with bases in the target sequence depending on the site of attachment of the dye on the oligonucleotide. Further structural studies are clearly needed to explain these observations.

The main conclusion from the studies presented above is that it is possible to target photochemical reactions to a specific nucleic acid sequence. This could be a useful method to introduce damages at a specific site on a single-stranded DNA template and therefore to induce site-directed mutagenesis. Photosensitized reactions can also be targeted to homopurine–homopyrimidine sequences in double-stranded DNA, provided the photosensitizer is attached to a homopyrimidine oligonucleotide. The latter binds in the major groove of the double helix, forming a local triple helix (Le Doan et al., 1987b; Praseuth et al., 1988). The reactions photosensitized by proflavin could also be induced if the target was a messenger RNA rather than a DNA fragment. Therefore, it should be possible to use light to artificially control mRNA translation in a specific way. It is very likely that photocross-linking of an oligonucleotide to a specific sequence on a mRNA, or other site-specific photosensitized reactions, would prevent mRNA translation.

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Registry No. **1**, 92-62-6; **2**, 74165-99-4; **3**, 113110-49-9; **4a**, 113110-50-2; **4b**, 113110-51-3; **5a**, 113110-53-5; **5b**, 113110-55-7; (5′)-d(TGAGTGAGTAAAAAAAATGAGTGCCAA)-(3′), 112603-07-3; T_8-Pf, 113132-22-2; Pf-T_8, 113132-23-3; Pf-[α]-T_8, 113159-77-6; α-octathymidylate, 11310-56-8; β-octathymidylate, 1270-05-9.

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Photoaffinity Labeling of *Escherichia coli* RNA Polymerase/Poly[d(A-T)] Transcription Complexes by Nascent RNA†

Thomas M. Stackhouse and Claude F. Meares*
Chemistry Department, University of California, Davis, California 95616
Received September 14, 1987; Revised Manuscript Received December 22, 1987

ABSTRACT: To elucidate the molecular interactions during transcription by *Escherichia coli* RNA polymerase, we have performed a quantitative analysis of the photoaffinity labeling produced by an aryl azide positioned at the leading (5′) end of the nascent RNA. Macromolecular contacts on the path of RNA across the transcription complex containing the template poly[d(A-T)] are observed as a function of the length of the transcript. Quantitative analysis provides the percent yield of photoaffinity labeling in the transcription complex by each length of RNA. Significant yields are observed for DNA, the β/β' subunits (analyzed together), and the σ subunit. The α subunit is not labeled under these experimental conditions. The DNA template is labeled by the leading ends of RNA molecules 5-18 bases long, with yields ranging from 1% to 6%. Photoaffinity labeling of poly[d(A-T)] is also observed for many transcript lengths longer than 18 nucleotides, but the yields are too low to quantitate. Labeling of the β/β' subunits occurs with ≈50% yields for transcripts of lengths ≥12 nucleotides; low but significant labeling yields of 1-8% by shorter RNAs (3-10 nucleotides) are observed. Labeling of the σ subunit is detectable for transcripts from 7 to more than 19 nucleotides long; quantitative measurements were possible up to the 19-mer. The RNAs most likely to be photoattached to the σ subunit are 9-12 nucleotides long, with a maximum photoaffinity labeling yield of 15% by the decanucleotide. These results modify the conclusions of previous work concerning the release of σ from an *E. coli* RNA polymerase/poly[d(A-T)] transcription complex [Hansen, U. M., & McClure, W. R. (1980) *J. Biol. Chem. 255*, 9564-9570]. The photoaffinity labeling of σ in poly[d(A-T)] transcription complexes differs from the results observed with DNA containing either the λ P_R or the T7 A1 promoter [Bernhard, S. L., & Meares, C. F. (1986) *Biochemistry 25*, 5914-5919], providing further evidence that the interaction between the nucleic acids and the σ subunit in the transcription complex depends on the nucleotide sequence.

The control of gene expression at the level of transcription has been studied most extensively with *Escherichia coli* RNA polymerase. This oligomeric enzyme (EC 2.7.7.6) catalyzes the synthesis of ribonucleic acid from a deoxyribonucleic acid template. RNA polymerase from *E. coli* contains five major subunits, with a total molecular weight of 449 000. The primary structures of all the subunits have been determined: α (M_r 36 512; Ovchinnikov et al., 1977); β (M_r 150 619; Ovchinnikov et al., 1981); β' (M_r 155 162; Ovchinnikov et al., 1982); and σ (M_r 70 263; Burton et al., 1981). The core enzyme contains four subunits ($\alpha_2\beta\beta'$) and is capable of elongating, but not efficiently initiating, RNA transcripts from promoter sites on DNA. Efficient initiation of a transcript at promter sites on DNA requires the holoenzyme, which contains the core RNA polymerase and the σ subunit. The presence of another subunit (ω) has also been observed [see Gentry and Burgess (1986) and references cited therein]; the function of ω is not yet established.

It was discovered by Travers et al. (1969) that σ can be released shortly after the initiation of transcription and subsequently σ can bind to another core enzyme to initiate another transcript. This is referred to as the σ cycle (Lewin, 1983). A detailed understanding of the mechanism of the σ cycle and its relation to the initiation reaction has interested many investigators (Chamberlin, 1974; Hansen & McClure, 1980; Shimamoto et al., 1986). It is believed that release of the σ subunit is accompanied by the formation of a stable elongation complex containing core enzyme, DNA template, and nascent RNA. The elongation complex could subsequently bind other factors involved in control of transcription and translation (McClure, 1985; Greenblatt et al., 1987). For example, it has been shown that nusA protein can bind to the elongation complex only after σ release (Greenblatt & Li, 1981).

In order to determine the RNA length at which σ is released, Hansen and McClure (1980) performed an elegant experiment measuring the amount of σ present in a mixture of *E. coli* RNA polymerase/poly[d(A-T)] transcription complexes containing various transcript lengths. By careful comparison of the amount of σ released with the lengths of RNA present, these authors concluded that σ is released quantitatively from the complex by the time a transcript of eight or nine nucleotides has been produced on poly[d(A-T)].

Other investigators have examined the release of σ from transcription complexes that contain the A1 promoter of bacteriophage T7 (Shimamoto et al., 1986). This group an-

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